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EDITOR'S NOTES

Several important science and math topics are examined in this special topics edition of the spring 2019 issue of the *Journal for the Liberal Arts and Sciences*. Featured are the works of researchers and educators from Ball State University, Oakland City University, the Zero Abuse Project, the Institute for Mathematics and Computer Science, University of Wisconsin-River Falls, and the Broward County Public Schools.

In an ever growing and highly mobile world, where food materials are shipped over vast distances, food safety issues have become especially significant, to the point of life and death in some cases. The first two articles relate different studies focusing upon food safety as it is impacted by bacteria. The first study involves a mini-review that outlines the basic background and biology of bacteriophage found in dairy milk. "By studying host range, DNA sequence analyses, and morphology," the researchers noted, "phage candidates may be identifiable with promise as agents for biological control of *Bacillus* spp. of concern to dairy scientists and consumers." Along the same lines of bacterial concerns in the food industry, the journal's second study focuses upon the use of phosphate buffers (in the presence of fructose) for inhibiting the growth of *E. coli* (which is a problematic contaminant of meats, fruits, and vegetables). Outside of the food industry, applications would include those pertaining to molecular diagnostics and treatments.

In the area of modern forensic science, another researcher's experiment focused "on the validation of real-time, forensic DNA analysis equipment and commonly used Short Tandem Repeat (STR) forensic DNA profile development chemistry." The researcher found that "the genotyping systems and applicable chemistries confronted through this validation study continue to remain dependable processes for forensic casework examination and prove that forensic DNA analysis truly remains one of the most well-studied and scientifically rigorous disciplines within the realm of forensic science."

A study concerned with the stimulated stress caused by video gaming is the topic of the journal's fourth article, "offering an analysis of video game genres in relation to stress reactions in players who already have a higher level of stress than average." The irony, the writer observed, is that many of the games examined "are often thought of as a stress reliever and players may not realize that the game is significantly affecting their stress reaction in a negative way until after the effect has begun."

As our environment comes under greater stress, the ongoing use of large landfills threatens the health of our planet. The next study in this issue of the *JLAS* addresses one aspect of the problem by looking at the use of geophysical techniques to monitor landfill leachate. The researchers call for particular action, pointing out, "With the potential for disaster so great in association with failure of fly ash and solid waste landfills, it is imperative that new, more cost-effective methods of monitoring leachate be implemented. The cost in cleanup, human and animal life, and environmental stability are too high to risk inaction."

The teachings of math and computer science are discussed in the last two articles of this special *JLAS* issue. The first study explores the possible relationship between gifted middle school students' images of a self-paced, challenging, process-based curriculum, Elements of Mathematics: Foundations (EMF), and their images of mathematics. The final writing is a short, but interesting essay of personal content concerning how far we may not have come in teaching girls to program in the computer science arena.

Kiev Gracias, Guest Editor
Journal for the Liberal Arts and Sciences

Bacillus spp. Bacteriophage and the Dairy Environment

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Abstract
Bacillus spp. are within a family of bacteria known to form spores for survival in extreme environments. The spores are resistant to temperature extremes, radiation, antibiotics, and many disinfectants. When ingested by or exposed to susceptible animal hosts, the spores can germinate and create biofilms or cause infections such as endophthalmitis, bacteremia, pneumonia, or gastroenteritis. The virulence potential makes these bacteria problematic in the food industry as pathogens and spoilers. Biological control of Bacillus spp. may be possible with bacteriophage. As a protective measure, dairy farmers could utilize a known bacteriophage after pasteurization to ensure dairy product quality and safety. This mini-review outlines the basic background and biology of bacteriophage found in dairy milk. By studying host range, DNA sequence analyses, and morphology, phage candidates may be identifiable with promise as agents for biological control of Bacillus spp. of concern to dairy scientists and consumers.

Introduction

Bacillus cereus

Bacillus cereus (*B. cereus*) is a Gram-positive, rod- shaped, aerobic- to facultatively anaerobic bacterial species (Bottone, 2010) that belongs to the *Bacillus cereus* group, which also consists of *B. anthracis*, *B.thuringiensis,* and *B. weihenstephanensis* (Helgason *et al*., 2000). These three bacterial species are very similar phenotypically. In fact, *B.thuringiensis* can only be differentiated from *B. cereus* by the presence of genes coding for insecticidal proteins (Cry toxins) in

the former. Cry toxins are crystalline structured proteins that have been used in commercial insecticides since the mid-1900s (Ibrahim *et al.,* 2010). *B. cereus* had long been regarded as simply a contaminant in clinical samples, and a spoilage organism in food products (Bottone, 2010). However, in the last 50 years*, B. cereus* has been found to cause a plethora of human infections. Presently, *B. cereus* is most commonly associated with gastrointestinal illnesses such as emesis and diarrheal syndrome. This type of intoxication or toxico-infection generally occurs through the ingestion of viable endospores or vegetative cells from contaminated food at densities at or above ~10^5/g.

The high-throughput nature of the dairy industry necessitates sensitive and selective assays to rapidly detect pathogens. According to the CDC, an estimated 48 million cases of foodborne illnesses occur each year. It is unclear how many cases occur worldwide due to the fractured nature of the data, as well as the prioritizing of particular foodborne organisms (Endersen *et al*., 2014). The presence of unwanted organisms in food not only poses a risk to public health, but also costs food industries millions of dollars a year due to food spoilage, or undesired physical changes to a food product that make it no longer edible. Even with modern preservation practices, approximately 25% of food is lost in the United States due to microbial spoilage.

The ubiquitous nature of *Bacillus cereus* combined with its ability to produce endospores that are highly adhesive makes these bacteria an ever present problem in food production, packaging, and shipment/storage (Lee *et al*., 2011). Furthermore, although the optimum temperature for a vegetative *B. cereus* cell is between 35 and 40^{o}C, the endospores formed by *B. cereus* are resistant to high temperatures and desiccation, allowing them to survive many quality control practices such as pasteurization and dehydration. Subsequently, even if pasteurization effectively kills all viable cells, when the process is complete the undamaged endospores germinate into viable cells that are able to cause disease.

B. cereus relies heavily on exoenzymes as well as enterotoxins to cause tissue destruction (Bottone, 2010). *B. cereus* has been found to produce three pore-forming

enterotoxins, and one heat stable emetic toxin. Hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K are secreted when vegetative cells, or endospores are ingested. Once released, these enterotoxins induce diarrheal syndrome. Conversely, the emetic toxin, termed cereulide, can be formed in food products, and excreted, thus *B. cereus* can still cause emesis even if no viable cells are ingested. *B. cereus* has also been found to cause infections of the lungs, eye, skin, liver, heart, central nervous system, and urinary tract using many of the same toxins.

Bacteriophages

Bacteriophages (phages) are viruses that have bacterial hosts. Phages are numerous and make up the largest group of viruses, having been detected in a myriad of natural environments (Ackermann, 2001). Phages exhibit a wide range of morphologies, including tailed, filamentous, cubic, and pleomorphic Phages are also characterized based on genomics (Table 1). Phage genomes may be either RNA or DNA, and either can be single stranded or double stranded. RNA phages can be further characterized by whether or not their genomes are segmented, or packaged in fragments within the capsid (Gottlieb *et al.*, 1990).

Table 1. Overview of bacteriophage families

Shape	Order or family	Nucleic acid, particulars, size	Member
	Caudovirales	dsDNA (L), no envelope	
	*Myoviridae*	Tail contractile	T4
	*Siphoviridae*	Tail long, noncontractile	λ
	*Podoviridae*	Tail short	T7
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2

From: Ackermann, 2007. C, circular. L, linear

Lytic vs Lysogenic Replication Cycles

Phages can be further grouped based on the type of replication cycle they undergo (Sulakvelidze *et al.*, 2001). For example, some phage can be virulent while others are termed temperate. If a phage is virulent, it is only able to undergo a lytic replication cycle, wherein the phage would attach to its bacterial host and inject its genetic material into the bacterium. Once inside, the phage hijacks the cell's machinery to translate phage proteins. Once the proteins are made, they are assembled within the host and the fully assembled phages are released through lysis of the host. The process then repeats when the mature phage recognizes another host cell, attaches, and enters. Phages can also be temperate. A temperate phage undergoes lysogenic replication.

Lysogenic replication differs from lytic replication in that the phage can undergo an alternate pathway that does not involve

immediate lysis of the host. The phage must still recognize and attach to its host bacterium, as well as inject its genetic material into the host. Once injected, the phage DNA may become incorporated into the bacterial host's genome, generating a prophage. If this happens, the bacterium can replicate normally many times until a specific environmental stressor occurs, triggering lysogenic induction, which leads back to the lytic pathway. Lytic phages are often easier to find, as they can be detected through simple plaque formation assays much more quickly than lysogenic phage (Figure 1).

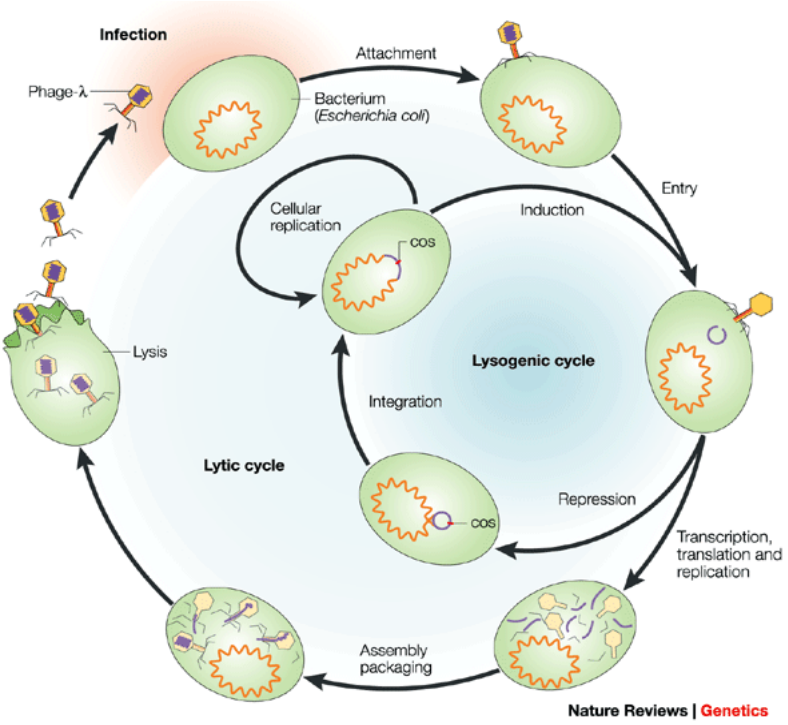

Figure 1: The lytic and lysogenic cycles of a lambda phage. The steps of these cycles are relatively conserved among most types of phages. (Adapted from Campbell, 2003)

Phage Therapy and Biocontrol

Phages also have a very specific host range, and are often only able to infect and lyse a single species or subspecies of bacteria (Lee *et al*., 2011). Because of this selective toxicity, they have been considered as a possible way to treat bacterial infections in humans and other animals. This treatment is called phage therapy and has been investigated essentially since phages were discovered (Sulakvelidze *et al.*, 2001). The selectively toxic nature of many phages means that not only would the patient receiving treatment not be affected, but nontarget beneficial bacteria would also be unaffected by treatment. The killing of nontarget commensal bacteria has long been a problem with many antibiotic treatments, making phage theoretically a better alternative in this instance. Another characteristic of phages that make phage therapy an attractive means to treat infection is their ability to co-evolve with their host, reducing the occurrence of phage-resistant bacteria. Phages can also be used to reduce the number of bacteria in areas outside of medicine, like the food industry. This type of treatment is termed phage biocontrol. In the past, phages as a therapy or as a biocontrol agent were studied, but with the emergence and spread of antibiotic resistance, interest in phages has increased (Brussow, 2005).

Phages are most effective when used in cocktails, which is a mixture of different phages that have the same host. Using a cocktail not only further reduces the risk of host resistance, but improves the efficacy of treatment by widening host range and the mechanism of lysis (Chan *et al.*, 2013). The phage based treatment Listshield™ is the first commercially available product to use a phage cocktail to treat *Listeria* in meat. Phage cocktails have also been used as biocontrol methods for other bacteria, such as *E. coli* in meat products (O'Flynn *et al*., 2004) and *Salmonella* in livestock (Wall *et al.*, 2010).

Setbacks and Concerns for Phage Therapy

Some concerns still exist when it comes to implementing phage control. The first is that many factors have an influence over the ability of phage to lyse host cells, i.e., temperature, pH, and bacterial density (O'Flynn *et al*., 2004). Even the slightest deviation from the phage's preferred conditions can result in an

ineffective treatment. Whether the phages are being used to treat humans, animals or animal products, these factors cannot always be controlled or predicted. Additionally, if the phage is a temperate phage, it is difficult to predict which cycle the phage will undergo, further making therapy less effective and predictable. Another concern is the unwanted transfer of virulence genes to host bacteria (Brussow, 2005). Many bacteria develop virulence factors as a result of the presence of a prophage that has been incorporated into the bacterial genome through lysogeny. It is possible that during the phage replication process, horizontal gene transfer could spread virulence factors. Both of the above-mentioned problems can be reduced if not eliminated, by carefully selecting the phages in each cocktail and not including any temperate phages.

There are also concerns with phage gene activity occurring within mammalian cells. Mammalian cells have also been found to take up phage DNA and incorporate it into their genomes (Brussow, 2005). When given phages orally, it was found that a small number of cells in the digestive tract of mice took up the phage DNA and could even incorporate it into their genomes. However, due to the vastly different cellular mechanisms between a eukaryotic cell and a bacterial cell, there is no indication that the intricate processes required for phage replication could occur in a eukaryotic cell.

Phages as a Detection Assay for Bacteria

Many of the same factors that make a phage an attractive candidate for the reduction of bacteria in an environment also make them a good candidate for detecting unwanted bacteria. The sensitive and selective nature of phages, as well as their sheer abundance and diversity make them a favorable tool for the detection of almost any kind of bacteria (Edgar *et al.*, 2006). Generally, most assays used to detect pathogenic bacteria are lengthy. This is due to the need for an enrichment and/or an amplification step to allow the bacteria to become more numerous and reach detectable levels. Due to the highly efficient nature of the food processing industry, time is valuable when testing for pathogens and food spoilage organisms. The sensitivity of most phages, when applied to a detection assay, could reduce the time needed to detect such bacteria. Phage

based detection assays already exist for bacteria such as *E. coli* O157:H7, *Mycobacterium tuberculosis*, and *Listeria.* Furthermore, phage based detection assays have been developed to detect spores of the highly infectious *Bacillus anthracis.* However, no literature has been found on a phage-based method to detect *Bacillus*; therefore, this area represents an open direction for applied research that would impact the dairy industry.

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Fructose-6-Phosphorane Inhibits *Escherichia coli* Growth

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Abstract
E. coli growth was inhibited by fructose in phosphate buffer media at pH 7 and above. Further analysis revealed a phosphate-fructose adduct, fructose-6-phosphorane, as the inhibitor. Fructose-6-phosphorane was isolated and was found to selectively inhibit E. coli growth; it did not selectively inhibit the growth of B. subtilis, S. aureus, and S. marcescens.

Introduction

Escherichia coli (*E. coli*) is one of the most studied prokaryotes in the microbial world. First discovered by the German microbiologist and pediatrician, Theodor Escherich, it is now known as the "biological rock star" (Zimmer, 2008) and has served as a model ("microbiological lab rat") for most studies on microbes as well as one of the first line of testing for new medicines. It is easy to find and work with and non-pathogenic strains that grow quickly on many different nutrients can be isolated from virtually any human (Blount, 2015). Studies on *E. coli* has led to some amazing breakthroughs in science including the genetic code, replication, transcription, and translation (Crick, n.d.). Even though most *E. coli* strains are nonpathogenic, there are some strains that are responsible for some ailments. For example, of the 265,000 estimated shiga toxin-producing, STEC, infections that occur annually in the USA, about 36 percent of these infections are caused by *E. coli* 0157:H7 *("E. coli* | NIH: National Institute of Allergy and Infectious Diseases," n.d.). Any new information to help in dealing with these infections or with

the utility of *E. coli* cells is a welcome help in fighting diseases and advancing science in general.

The utilization of buffers for cell culturing is vital for studies with pH requirements (Will et al., 2011). Phosphate buffers are particularly important as they provide the required pH, serve as a source of inorganic phosphates, and are necessary for controlling osmolarity of cells. Phosphate buffers at pH 7 and above (e.g. PBS) are commercially available. In most cases, the ability to form insoluble salts with several biologically important cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, and Co^{2+}) is the only limitation to their use (Williamson and Cox, 1968). Inorganic phosphates are essential for the growth of bacteria (Kuo et al., 2013). Their availability is sometimes a factor for the sustenance of some species of bacteria. In recent times, agricultural activities have increased the availability of organophosphates especially in soil. As a result, certain bacteria have adapted to feeding on these organophosphates which in turn help degrade their toxicity (Singh, 2008).

In an experiment to determine the effect of the metabolism of the glycosidic bond on the rate of growth of *E. coli* culture, we observed inhibition of *E. coli* at pH 7 and above when cultured in phosphate buffers in the presence of fructose.

Materials and Methods

The experiment was designed to determine *E. coli* metabolism of the two main components of sucrose at varying pH by indirect monitoring of cell growth rate. Since we wanted a buffer that could provide acidic, neutral, and basic pH, phosphate buffer solutions were utilized. Three 0.2 M phosphate buffer solutions at pH 6, 7, and 8 were prepared. Each buffer was divided into four 20 ml portions. To the first portion, 0.684 g of sucrose was added to make a 0.1M solution. To the second, 0.72 g glucose (0.2 M); to the third, 0.72 g fructose (0.2M); and finally, to the fourth, a combination of 0.36 g each of glucose and fructose (0.1M glucose + 0.1M fructose). *E. coli*, *B. subtilis*, *S. aureus*, and *S. marcescens* (Carolina) were each cultured in all twelve solutions (Table 1) and monitored.

Table 1. Sugar-Phosphate buffers for bacterial cell culture.

pH	6 A	7 B	8 C
1	0.1M Sucrose [a]	0.1M Sucrose [a]	0.1M Sucrose [a]
2	0.2M Glucose	0.2M Glucose	0.2M Glucose
3	0.2M Fructose	0.2M Fructose	0.2M Fructose
4	0.1M Glucose+ 0.1M Fructose	0.1M Glucose+ 0.1M Fructose	0.1M Glucose+ 0.1M Fructose

[a] Since sucrose is a chemical combination of glucose and fructose, we wanted to see if 1M sucrose has a similar effect as 1M fructose + 1M glucose

Results and Discussion

After two months of culturing, while all others showed varying levels of growth in the cultures, no growths were observed in the cultures of pH 7 and above containing fructose (B3, B4, C3, and C4; please see Table 1). Instead, the cultures turned a clear yellow. However, we did not observe this inhibition in fructose solutions of non-phosphate buffers at similar concentrations and pH.

After a few control experiments were conducted to eliminate other options, we concluded that a fructose-phosphate adduct (which was forming) was responsible for the inhibition. The adduct was later found to be fructose-6-phosphorane. Similarly, we detected glucose-6-phosphorane in the glucose cultures of pH 7 and above (although that caused no inhibition). Although inhibition of *E. coli* by fructose and fructose-1-phosphate had previously been reported as a consequence of mutation (Ferenci and Kornberg, 1973), no report of inhibition by fructose-6-phosphate exists.

With the isolation of the solid sodium salts of fructose-6-phosphorane, *E. coli* cultures were grown in commercial EC media, deionized water, and 0.2M glucose solution (each with 0.2M concentration of the salt). As controls, the same cultures were prepared without the salt. *E. coli* growth was inhibited in all the solutions with the salt, but not in the controls. However, when *B. subtilis*, *S. aureus*, and *S. marcescens* (Carolina) were cultured under similar conditions, no inhibition was observed. In fact, we observed a relative increase in growth for *S.*

marcescens (which is known to feed on phosphates) (Abo-Amer, 2011). No inhibition was observed when the salt concentration was reduced to 10 μM in commercial EC media.

Contrary to most bacteria, *E. coli* has been shown to metabolize fructose in three separate pathways other than the glucose pathway (Ferenci and Kornberg, 1971; Kornberg, 2001). It is possible that fructose-6-phosphate is an inhibitor for one of the non-glucose pathways. At pH 7 and above, monosaccharides and other soluble hydroxyl ketones and aldehydes in phosphate buffers are not available as free molecules, but as phosphoranes. This has huge implications for cell culturing in phosphate-based media. If it is deemed undesirable, then the use of phosphate buffers for cultures containing hydroxyketones and hydroxyaldehydes may have to be re-examined.

Conclusion

In conclusion, monosaccharides form adducts with inorganic phosphate at pH 7 and above. Fructose-6-phosphorane (the fructose adduct) has been shown to inhibit *E. coli* growth in this bacterial cell culture experiment. Considering the huge role of organophosphates in agriculture, medicine, fire retardants etc., we are re-examining the biophosphorylation process thoroughly. The success of this research will also help create a working knowledge for applications in gene therapy, protein function, and treatment of diabetes.

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Pushing the Envelope: An In-Depth Validation Study of Modern Forensic Biology Equipment and Chemistries

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Abstract
This experiment focused on the validation of real-time, forensic DNA analysis equipment and commonly used Short Tandem Repeat (STR) forensic DNA profile development chemistry. In particular, the reliability of the Applied Biosystems® Quantifiler Trio DNA Quantification Kit, with the Applied Biosystems® 7500 Real-Time PCR System with HID Real-Time PCR Analysis Software v. 1.2 and the Promega® PowerPlex Fusion 6C System, alongside the Applied Biosystems® 3500 Genetic Analyzer with GeneMapper ID-X Software v. 1.4 were subjected to a wide variety of samples that mirror actual forensic casework samples across a wide spectrum of qualitative and quantitative variances. The findings of the study, which include samples and results pertinent to environmental contamination, substrate influence, low DNA quantity, DNA mixtures, mock evidence, species of origin challenges, and precision assessment were examined in-depth. Based upon the data generated from the study herein, pontifications regarding the reliability and trustworthiness of modern forensic DNA methods and instrumentation should be refuted with little discourse and questionable forensic practices with little scientific rigor or sufficient data supporting the results thereof should be the focus of future scrutinizing research seeking to inject questionability into the discipline.

Introduction

The following narrative details the results of a comprehensive study designed to challenge current forensic

DNA extraction, quantitation, polymerase chain reaction (PCR) and capillary electrophoresis (CE) equipment and Short Tandem Repeat (STR) forensic PCR kit performance through a series of experiments aimed at mimicking realistic casework samples and the results thereof. Specifically, this study was created in such a way as to validate the robustness and performance of the Applied Biosystems® Quantifiler Trio DNA Quantification Kit (using the Applied Biosystems® 7500 Real-Time PCR System with HID Real-Time PCR Analysis Software v. 1.2) and the Promega® PowerPlex Fusion 6C System (using the Applied Biosystems® 3500 Genetic Analyzer with GeneMapper ID-X Software v. 1.4). Given that forensic science has come under fire for lacking scientific rigor and quantitative data to prove that certain disciplines are in fact "true science," with the most recent challenge to the field culminating through the release of the Presidential Council of Advisors on Science and Technology (PCAST) 2016 report that specifically targeted the scientific validity of various forensic techniques, it was the aim of this study to supplement and support the historic body of research proving that current, up-to-date chemistries used in forensic DNA analysis are reliable, accurate, and worth continued use for fact development as it pertains to courtroom testimony and criminal case resolution. Performing such studies are necessary to counteract reports that cast doubt on forensic science as a valuable field of investigative inquiry in the Criminal Justice system. Validation studies such as the one herein are in accordance with the recommendations of the Scientific Working Group on DNA Analysis Methods (SWGDAM). The SWGDAM validation guidelines have been crafted around the FBI's Quality Assurance Standards (QAS) and thus aim to assist laboratories in validating procedures that are aligned with the aforementioned standards (SWGDAM Validation Guidelines, 2016). Validation studies are important so as to test the limitations and reliability of the procedures used in a forensic laboratory. The major experiments performed in this endeavor include an environmental insults study, a substrate study, a minimum sample study, a mixture sample study, a mock evidence (casework) study, a precision study, and a species specificity study. The results obtained from each respective study are then used in an informative manner to assess a wide range of

theoretical casework sample scenarios that not only challenge the chemistries and systems in place at many forensic science laboratories across the U.S., but that also test the limits of an analyst's training with respect to DNA extraction, quantitation, and analysis.

Materials and Methods

Insult Study

The goal was to examine DNA quantity and quality via Quantifiler Trio, Fusion 6C systems after exposure to environmental insults, chemicals that mirror casework evidence. As a result, various insults were added to a sterile cotton-tipped applicator (FisherBrand, catalogue number 23-400-115; Marshalltown, IA) and allowed to dry when applicable. The following list of insults was used: sandy soil (Washington, IN source), dark soil (Washington, IN source), U.V. exposure (UVP Crosslinker, AnalyticJena, model CL-1000; Upland, CA), high heat exposure (Corning stirrer/hot plate; Corning, NY), phenolphthalein (in-house reagent preparation), luminol (Evident Crime Scene Products, item number 3176-8; Union Hall, VA), silver/black dual purpose latent print powder (Tri-Tech Inc., order number LP-SB-2; Leland, NC), ninhydrin (3M Novec, HFE 7100; St. Paul, MN) commercial glass cleaner (Great Value, item number 552048401; Walmart Inc., Bentonville, AK), and hand soap (Dial, item number 554754617; Walmart Inc., Bentonville, AK).

Human blood was diluted 1:10 using a known source and nuclease-free water (Promega, reference number P119C; Madison, WI) and a small amount (~100 microliters [uL]) was added to each insulted applicator. In the case of the U.V. exposure, the blood-wetted applicator was exposed to ten minutes of the 254 nm U.V. source in the UVP Crosslinker instrument. In the case of high heat exposure, the bloodied applicator was held next to the hot plate surface while the maximum heat value was selected on the dial of the instrument (Karni *et al.*, 2013). The applicator was slowly rotated over ten minutes to allow for even heating of the sample on the applicator.

A total of fifteen samples were created from the list of insults above. Ten samples were processed using Maxwell® 16

automated extraction (Promega, reference number AS4600) and the Maxwell automation extraction kit (Promega, reference number AS1130) by way of Maxwell regular extraction protocol (Indiana State Police Section 2.8.10.1, 2017). Given this manual applies to actual casework methods for forensic evidence analysis, the author determined the procedures therein to be a suitable set of methods by which to parallel in the body of this study. To compare DNA yields from alternative extraction methods, five samples were concurrently extracted using regular organic DNA extraction (phenol-chloroform-isoamyl alcohol; Fisher, item number 108-95-2; Lenexa, KS) with Microcon concentration (Millipore Sigma, item number MRCF0R100; Billerica, MA; Indiana State Police Section 2.8.4, 2017). Samples extracted were then quantified using the Applied Biosystems ® Quantifiler® Trio chemistry (Fisher, item number 4482910) with the Applied Biosystems® 7500 Real-Time PCR System (Fisher, item number 4359284). Results of the quantification were determined using the HID Real-Time PCR Analysis Software v. 1.2 (Fisher, item number A24664). Amplification of samples was performed using the Promega® PowerPlex® Fusion 6C chemistry (Promega, reference number DC2705) with the Applied Biosystems ® 3500 genetic analyzer (Fisher, item number 4406017) being used for electrophoretic separation of amplified DNA targets. Results in the form of an electropherogram were examined using the GeneMapper ID-X Software v. 1.4 (Fisher, item number 4479715). Quantifiler Trio (Indiana State Police Section 2.8.4, 2017), Fusion 6C amplification and 3500 genetic analyzer methods were used simulated forensic evidence processing use.

Substrate Study

The rationale for this segment of the study was to evaluate the possible effects of substrates (or components thereof) on body fluid DNA analysis. Various body fluids were applied to an assortment of substrates (wood, leather, denim, glass) in both single source and mixed body fluid applications (Substrate Study Results and Discussion, Table 1). With respect to sample deposition on the substrates: 100 uL of undiluted blood was used on the single source samples. 100 uL of undiluted semen was also applied to single source substrate samples. Mixture

samples were made by combining 200 uL of the applicable body fluids into a sterile tube, vortexed gently, and 100 uL of the body fluid mixture was then applied to each applicable substrate. All stains were allowed to air dry before extraction.
Samples were harvested from each substrate (except for denim) using a nuclease-free water soaked sterile cotton-tipped applicator. Denim samples, due to the absorptive nature of the material, were taken via direct cutting using 10% bleach, 70% ethanol sterilized scissors. Once the samples were dried, cuttings were taken using sterile scissors for Maxwell® 16 automated extraction using the Maxwell automation extraction kit. Quantitation was carried out using the Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System. Amplification of samples was performed using the Promega® PowerPlex® Fusion 6C chemistry with the Applied Biosystems ® 3500 genetic analyzer being used for electrophoresis.

Minimum Sample Study

The purpose of this element of the study was to assess the sensitivity of the Promega® PowerPlex® Fusion 6C amplification kit and detection limits of the Applied Biosystems® 3500 genetic analyzer with varying amounts of input DNA. The 2800M Control DNA from the Fusion 6C kit was used as a known source of male DNA to be serially diluted for the purpose of this study. The 2800 M Control DNA was originally quantified via the Quantifiler Trio system to determine true concentration of the stock source prior to serial dilution. Serial dilutions (data not shown) were then made from this stock DNA and nuclease-free water to create samples at the following total DNA concentrations: 4.00 nanograms (ng), 2.00 ng, 1.50 ng, 1.00 ng, 600 picograms (pg), 400 pg, 200 pg, 100 pg, 60 pg, 20 pg, and 10 pg. Replicate samples were analyzed for 100 pg and 60 pg samples for concordance purposes.

Quantitation was carried out directly from each dilution sample using the Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System. Sample amplification and detection was likewise achieved using the Promega® PowerPlex® Fusion 6C chemistry with the Applied Biosystems ® 3500 genetic analyzer. The analytical threshold of

the 3500 genetic analyzer was 150 Rfu, while the stochastic threshold was 750 Rfu; these values for preliminary peak (allele) evaluation in electropherogram profiles for each dilution were determined via validation studies performed on the instruments used in this study (data not shown).

Mixture Sample Study

A hallmark of forensic samples is the ability to recognize samples consisting of complex DNA mixtures. As such, known two person (2P) and three person (3P) mixture data was created across varying ratios of DNA contributors in a sample series. Buccal swabs from two male sources and one female source were collected for this study. These swabs were then sampled and extracted via Maxwell® 16 automated extraction and the Maxwell automation extraction kit, and quantified using the Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System. The extracts from all three individuals were then used to make mixture samples of 2P and 3P proportions using nuclease-free water. The samples also varied in the amount of contributor DNA concentrations. The exact setup and concentration of each DNA contributor can be found in Table 1 and Table 2 below (see Mixture Sample Study Results and Discussion). Quantitation of the mixture samples was then carried out using the Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System. Amplification of 2P and 3P samples was achieved with the Promega® PowerPlex® Fusion 6C chemistry with the Applied Biosystems ® 3500 genetic analyzer being used for electrophoresis.

Casework Study

This sub-section of the study examined the Maxwell® 16 automated extraction with the Maxwell automation extraction kit, quantification of Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System, and amplification/electrophoresis of simulated evidentiary casework with the Promega® PowerPlex® Fusion 6C chemistry and the Applied Biosystems ® 3500 genetic analyzer. The samples analyzed were non-probative casework samples provided by a local law enforcement agency that included two sterile, cotton-tipped applicators used to swab from fingernail scraping picks

(one swab per pick) from the left and right hands of the hypothetical victim. In addition, the following samples were processed from the kit: two vaginal/cervical swabs; 2 external genital swabs; 2 dried secretion swabs (reported victim had been "kissed/licked" on item envelope); 2 anal swabs.
With respect to extraction, Maxwell automated extraction was performed on all samples, but different procedures were performed based on sample type and the potential for samples to contain mixed DNA profiles from the victim and suspect. The vaginal/cervical swabs, external genital swabs, internal genital swabs, and anal swabs were processed using differential extraction methods as a result of their potential to contain DNA mixtures (Indiana State Police Section 2.8.10.2, 2017). All remaining samples were extracted using regular Maxwell extraction methods previously referenced (Indiana State Police Section 2.8.10.1, 2017). Quantification was carried out on all extracts using the Quantifiler® Trio chemistry and the Applied Biosystems® 7500 Real-Time PCR System. Amplification was performed using the Promega® PowerPlex® Fusion 6C chemistry with the Applied Biosystems ® 3500 genetic analyzer being used for electrophoresis.

Precision Study

The purpose of this portion of the study was to evaluate the base pair resolution, sizing capability, and reproducibility of PowerPlex® Fusion 6C system for forensic casework analysis. 16 wells were loaded into a capillary electrophoresis optical plate using the Fusion 6C allelic ladder included in the chemistry kit. 1 uL of allelic ladder was loaded with 10 uL Hi-Di formamide (Life Technologies, catalogue number 4311320; Thermo Fisher Scientific, Indianapolis, IN). The Applied Biosystems ® 3500 genetic analyzer was used for electrophoresis using an injection protocol of 1.2kV/15s.

The following data was analyzed (via Microsoft Excel) from the study samples: minimum allele size (data not shown), maximum allele size (data not shown), mean allele size (data not shown), standard deviation of allele size, mean standard deviation of a locus, minimum standard deviation of a locus, and maximum standard deviation of a locus.

Non-Human (Species Specificity) Study

This part of the study examined the potential for non-human species interaction with Quantifiler® Trio and Promega® PowerPlex® Fusion 6C chemistries. Samples were taken from bloodstain identification cards saturated with undiluted blood from various animal sources. The following non-human sources were analyzed in the study: bovine, swine, canine, feline, and cervid (deer). Cuttings from each non-human source bloodstain card were then processed using Maxwell® 16 automated extraction and the Maxwell automation extraction kit and quantified using the Quantifiler® Trio chemistry with the Applied Biosystems® 7500 Real-Time PCR System. Amplification was performed using the Promega® PowerPlex® Fusion 6C chemistry with the Applied Biosystems ® 3500 genetic analyzer being used for electrophoresis.

Results and Discussion

Insult Study

Table 1 contains the results from quantification of the insult samples. All applicable controls for this study passed (data not shown). The Degradation Index (DI) value can be used in conjunction with Internal Positive Control (IPC) Cycle Threshold (Ct) values to determine sample quality. In accordance with the Quantifiler Trio User Guide, the DI value is used to help analysts ascertain the degree of degradation of the DNA extracted from a given sample (Applied Biosystems, 2017). Such information is important in determining the overall quality of the template to be amplified and can help resolve issues with poor profile resolution or incomplete profile resolution in downstream analyses. A DI value <1 indicates the sample DNA is not degraded, while a DI value ranging from 1-10 indicates slight to moderate DNA degradation, and any DI value >10 is correlated to significant degradation. With respect to the IPC Ct values, a Ct value is a relative measure of the cycle in the amplification process at which one can detect true DNA amplification from background noise natural to the polymerase chain reaction (PCR) process (Applied Biosystems, 2011). Thus, each sample contains an IPC consisting of synthetic DNA to assess the potential for inhibitor presence; if excess DNA or an inhibitor makes it through the extraction process, the IPC Ct value for a sample will

theoretically be higher, indicating that a longer amount of cycles were needed to result in observable DNA amplification.
With respect to DI values, most samples predominantly demonstrated limited degradation. Samples insulted with bleach, luminol, ninhydrin, and hand soap demonstrated values <1 for the DI, while sandy/dark soils, U.V. exposure, latent powder, and glass cleaner samples demonstrated slight to moderate degradation. Figure 1 demonstrates these values in an illustrative figure, accounting for both methods of DNA extraction. Dark soil, rich in humic acid, appears to be one of the higher DI-laden sample types regardless of extraction method, which is logical given its ability to interfere with DNA polymerase inhibition via attachment to the enzyme or by way of blockage of polymerase reading activity through binding directly to the DNA template (Matheson *et al.*, 2010). U.V. exposure is another insult type that resulted in higher DI values regardless of extraction method. The degradation observed here is also anticipated given U.V.'s ability to dimerize thymine bases, leading to double-stranded molecules being rendered inseparable for PCR synthesis (Visser *et al.*, 1999). The only sample to experience high-level degradation was the applicator exposed to high heat (DI: 12.9). Given the use of high heat denaturation in PCR applications, it is sensible to consider that exposure to high heat over a consistent amount of time would ultimately lead to thermal degradation of covalent bonds within template DNA strands (Karni *et al.*, 2013).

DI has a direct impact on fluorescence values obtained from the electrophoretic detection process, as demonstrated in Figure 2. As shown in the aforementioned figure, the lower the DI value for a sample, the larger the fluorescent peaks; such data is indication that high quality DNA was amplified to greater success that led to better profile resolution for those samples. Figure 3 also demonstrates minimum peak intensity as it relates to DI values for this portion of the study. Again, the lowest allele peaks observed are correlated to higher DI values, thereby reinforcing the impact degraded DNA has on overall profile quality. The impact on larger molecular weight DNA targets from the Fusion 6C kit is demonstrated in Figure 4. As the figure illustrates, smaller molecular weight loci tended to amplify more successfully (higher relative fluorescence unit [Rfu] values) than

their larger sized counterparts. Such an observation is expected given the plethora of studies examining the increased susceptibility to degradation with large DNA fragments (Applied Biosystems, 2017; Smith *et al*., 2015).

With respect to IPC Ct values, the average standard IPC Ct value for the samples was 28.8. When comparing the average IPC Ct values to each insult sample, the only specimen to demonstrate any possible inhibition with the PCR process was the Maxwell extraction bleach sample, with an IPC Ct above average (29.3). Bleach has been observed to degrade DNA in blood-based samples (Passi *et al*., 2012), leading to poor profile resolution and reduced PCR efficiency; in fact, this degrading effect has even been known to increase with continued exposure to this insult (Harris *et al*., 2006). Thus, the heightened IPC Ct value observed could result from the degradation of DNA by bleach exposure, resulting in the observed increase in Ct due to less template being made available to amplify over the cycle span of the PCR reaction.

In regards to exposure of environmental inhibitors, it should be noted that this particular study honed in on one possibility as it pertains to insult exposure—inoculation of body fluid to a previously insulted surface. Further studies should focus on alternative exposure strategies; for instance, other studies have applied chemical insults directly to body fluids, which is the inverse of this study (Harris *et al*., 2006; Gross *et al*., 1999; Tobe *et al.*, 2007). The results from these studies were similar to those from the current study, where high molecular weight target degradation and minimal PCR reaction inhibition was observed. As a result, these insult studies appear to demonstrate consistent issues with insult exposure and the potential for minimal PCR inhibition. The weak impact of insults on the amplification capabilities of these samples could be related to the extraction process. A study by McCord *et al.* (2011) inoculated DNA extracts directly with various known inhibitors and found that direct, post-extraction interaction with environmental insults had a greater impact on PCR reaction efficiency. As such, it appears that the extraction process is able to concentrate DNA from evidentiary samples while successfully diluting insults in the final DNA extraction.

For an examination into the differences between extraction methods, refer to Figure 1 and Figure 5. DI values were consistently higher values for organic extraction. Organic extraction involves the use of harsh chemicals and more agitation-laden processes (e.g. several centrifugations, vortex steps), possibly leading to higher degradation values compared to the use of repeat washing and magnetic bead purification steps as observed via Maxwell robotic extraction. Overall, Maxwell extraction also demonstrated greater success in terms of insult DNA quantity regardless of samples processed by each extraction method, but organic extraction led to the highest yield of DNA from the study. Given that Maxwell magnetic beads can become saturated with DNA (Lim *et al*., n.d.), the organic method (which is limited by the amount pipetted from the upper-aqueous phase containing DNA) could inherently lead to greater DNA yields. The fact that the Maxwell extraction samples tended to succeed moreover in terms of higher yields versus organic extraction on identical samples could be attributed to the fact that the magnetic beads are more efficient in DNA retrieval and purification, whereas organic extraction could lose DNA through pipetting variability and repeated Microcon washes. Note that the DI is simply a comparative analysis of large molecular weight DNA amplification efficiency versus the smaller weight target amplification efficiency when amplified with the Quantifiler Trio chemistry. As such, the DI values may be inflated due to inhibitors making it through extraction and impacting larger molecular weight targets and not reflect actual degradation of target molecules. In summation, this segment of the study demonstrates the trade-off between extraction methods: Maxwell extraction yields consistent amounts of DNA with lower DI values, while more DNA (albeit degraded or inhibited to a greater extent) can be yielded from the traditional organic method. The reason for the higher DI values with organic extraction could be due to increased agitation procedures or an increase in inhibitors being carried over into the DNA sample, whereas Maxwell extraction minimizes inhibitor carryover to quantification since magnetized glass beads are carrying the DNA to extraction and leaving inhibitors behind in liquid through the various washes.

In closing, this study demonstrated the impact of insults on DNA quantity and quality as demonstrated from quantification and amplification/electrophoretic results. These environmental insults had minimal impact on sample degradation and amplification efficiency, with only a few insults creating pertinent issues involving template integrity. While these insults have a known history with respect to inhibition of the PCR process (Watterson, Blackmore, and Bagby, 2006; Bessetti, 2007), it appears that the current methods for extraction, quantification, and amplification are sufficient enough to mitigate most issues related to insult incorporation into casework samples.

Table 1. Quantitation Results for Insult Study Samples.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15
Insult:	Sandy soil	Sandy soil	Dark soil	Dark soil	UV	UV	Bleach	Bleach	Luminol	Luminol	Ninhydrin	Latent Powder	Heat	Hand Soap	Glass Cleaner
Extraction Method:	Organic	Maxwell	Organic	Maxwell	Organic	Maxwell	Organic	Maxwell	Organic	Maxwell	Maxwell	Maxwell	Maxwell	Maxwell	Maxwell
DI Value:	1.45	0.860	2.81	1.22	2.37	1.42	1.33	0.650	0.880	0.920	0.890	1.13	12.9	0.960	1.01
Total DNA (ng/uL):	0.153	0.245	0.397	0.739	0.471	0.0422	1.67	0.974	0.515	1.03	1.08	1.45	0.142	0.859	0.575
IPC Ct Value:	28.4	28.4	28.7	28.3	28.0	28.1	28.5	29.3	28.5	28.6	28.3	28.3	28.0	28.4	28.4
Sample Avg. Peak Height:	4507	9893	6709	7593	6641	1231	12554	15312	9950	5255	13297	6175	1446	14254	10052
Sample Min. Peak Height:	1085	6880	2220	3132	1346	206	7716	10923	5233	2277	6604	3191	153	7665	5355
Average DI for Insult:	1.16		2.02		1.90		0.990		0.900						

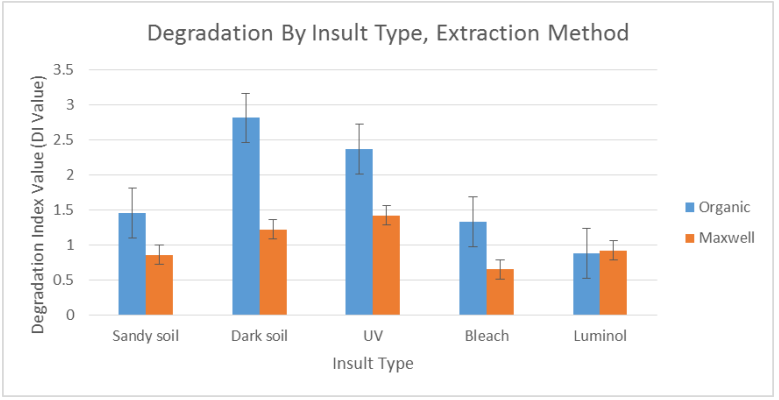

Figure 1. Degradation Index (DI) Values by Insult and Extraction Method.

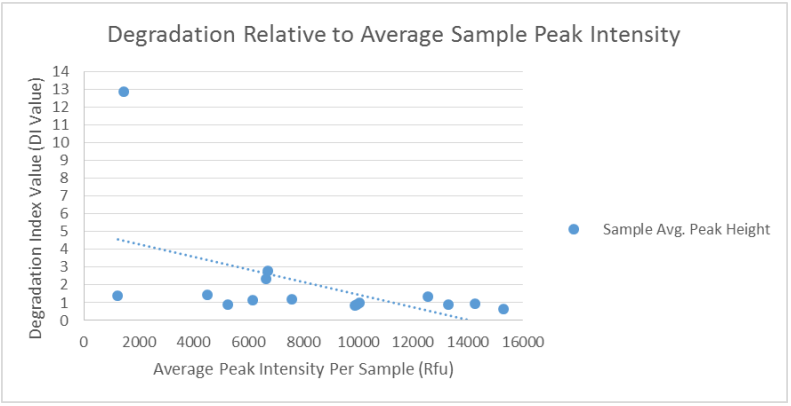

Figure 2. Impact of Degradation on Average Sample Peak Intensity.

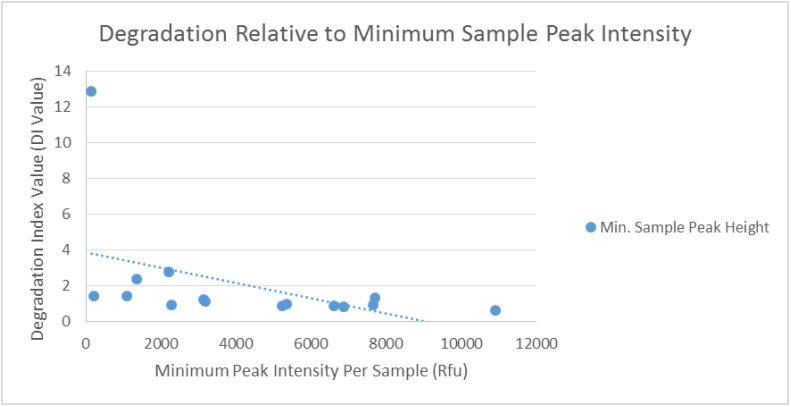

Figure 3. Impact of Degradation on Minimum Sample Peak Intensity.

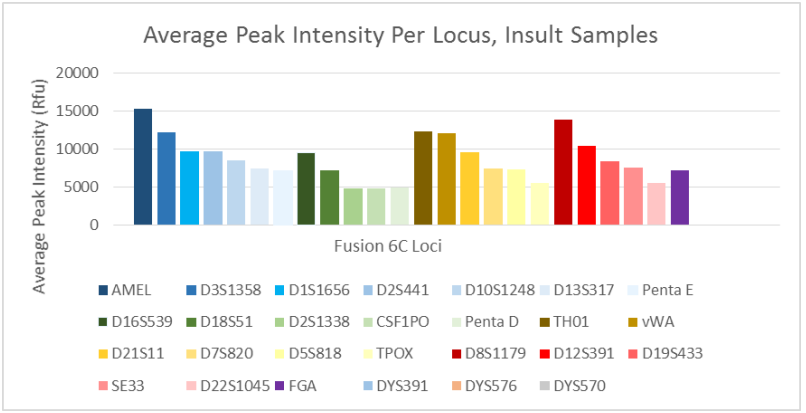

Figure 4. Average Loci Peak Intensity, Insult Samples.

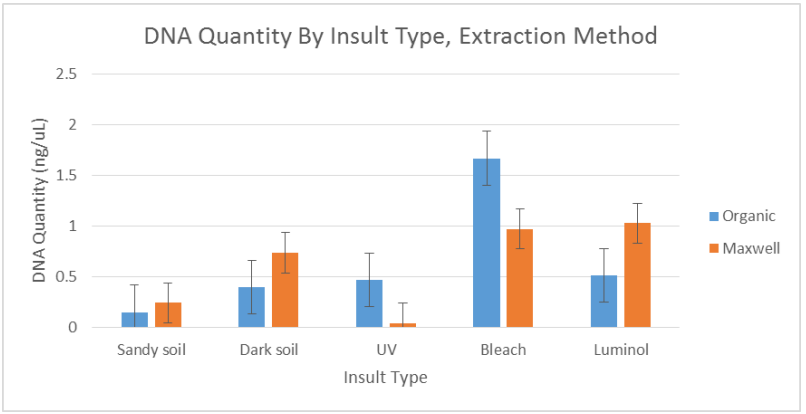

Figure 5. Insult Type and Extraction Method Impact on DNA Quantity.

Substrate Study

With the discovery of more sensitive DNA detection methods, such as the Fusion 6C system, came the ability to process minute amounts of DNA from small stains or touch DNA items, the latter of which is comprised of genetic material from epithelial cells left behind from touching or handling an object (Scientific American, 2017). Because smaller amounts of DNA are being processed today, the impact of substrates on PCR inhibition has become a major concern in forensic laboratories, especially with the potential for co-extraction of inhibitors

alongside pertinent DNA profiles from an item of evidence (Wang *et al*., 2008). Given that various substrates such as wood are rough and can trap DNA from stains and contact, or that denim or other assorted fabrics can soak up and trap body fluid stains, it is important to consider how chemicals inherent to these surfaces or used to treat these substrates can interact with the PCR process and applicable reagents (Ryan, 2012).

A wide range of substrates have been found to be chemically altered or intrinsically contain potential PCR inhibitors (Bessetti, 2007). Wood has been found to contain inhibitive compounds in a study by Lee and Cooper (Lee and Cooper, 1995). More specifically, acidic plant polysaccharides have been discovered that pose a threat to DNA amplification and can make their way past some extraction methods (e.g. organic extraction), and phenol-based compounds and humic acids have been found in plant matter that can lead to inhibition, as well (Bessetti, 2007; Saunders and Parkes, 1999). Dyes, such as indigo dye from denim fabric, have been found to inhibit PCR, as well (Larkin and Harbison, 1999). Dyes are problematic to the DNA detection process specifically in electrophoretic separation via capillary electrophoresis; specifically speaking to indigo dye, this blue-colored compound can compete with the fluorescent dyes used to detect the amplified DNA targets, leading to failed profile development from a sample (Opel *et al*., 2009). Leather is yet another challenging substrate that can be encountered from a forensic evidence standpoint. The tannic acid found in leather is known to bind to DNA polymerases, thereby inhibiting the PCR reaction as a result (Bessetti, 2007; Alaeddini, 2012). As one can discern, there are many inhibitors readily available on a given surface that can pose a threat to PCR and DNA profile development success.

Table 2 and Table 3 display the data resulting from substrate sample quantification for both the single source samples and the two person mixture samples. All applicable controls for this study passed (data not shown). With respect to degradation, the DI values for all samples (single source, two person mixture) reveal minimal degradation with respect to sample DNA. Rather large quantities of DNA were retrieved from the samples, as well, indicating that the substrates utilized in this study presented little challenge in terms of sample retrieval. With an average

standard IPC Ct value of 28.8, only one sample demonstrated slight inhibition in terms of when observable amplification from background noise was detected. The two person mixture with saliva and blood was found to contain the largest amount of DNA (ng/uL) of any sample in the study, which appears to be the likely culprit for the heightened IPC Ct value since excess DNA is known to have an inhibitory effect on the amplification process (Applied Biosystems, 2011). It should be noted that the saliva and blood sample also demonstrated a high female to male ratio value, indicating (alongside the low Y-chromosome target value; data not shown) that this sample suffered from poor mixing of the body fluid profiles. There are several reasons this phenomenon may be observed in the sample data, including: incomplete mixing of the body fluids prior to application on the substrate; different DNA concentrations from each body fluid in a mixture; and possible variation in body fluid contributor concentration with respect to sample cutting taken for DNA analysis.

Tables 4-7 examine the standard deviations, means, and coefficients of variance for study samples based on replicate sample analysis. Sample variance was then observed with regards to body fluid type and substrate type to determine if a given substrate could have significantly influenced degradation or DNA quantity from extraction. As the tables demonstrate, the standard deviation (a measure of variation between replicate samples) alongside the coefficient of variation (which uses the standard deviation and mean values for replicate samples) demonstrates that the samples demonstrated low variability in terms of degradation and DNA quantity retrieved from a given substrate or body fluid, indicating that there was no significant inhibitory issue detected with respect to the substrates or body fluid types examined in this study (Ready Ratios Standard Deviation, 2017; Ready Ratios Coefficient of Variation, 2017). Figure 1 is an illustration of the DI values by substrate. The overall DI values indicate that none of the samples were degraded to any significant degree based on the low values reported (Applied Biosystems, 2017). Figure 2 demonstrates DNA quantity by substrate. This figure reveals that leather had the highest amount of DNA retrieved from it, which (as explained earlier) could be the result of differential contributor DNA concentration in the sample taken for processing, sample size

concentration, etc. Denim demonstrated the lowest amount of DNA retrieved from the study materials. Given the composition of this material, it is probable that the sample was intertwined into the fibers of the fabric, making extraction more difficult as opposed to the other samples, where the body fluids were readily retrievable from the surface using a cotton-tipped applicator for greater body fluid collection. Arguably, the most informative information with respect to substrate influence on DNA analysis could be observed in Figure 3. This figure reveals that the glass substrate (sterile microscope slide) had the highest DNA amplification values; denim and leather substrate samples demonstrated lower fluorescence values for the DNA collected from these sources. As such, the lower fluorescence values for these samples correlate with concerns regarding indigo dye and tannic acid PCR interference, respectively. Given that there was minor variation between the substrate fluorescence values, the issue with co-purification of substrate inhibitors should be noted, but the consistency of the fluorescence values irrespective of substrate and fluid type indicates that current extraction methods employed are sufficient in mitigating the potential for co-purification of major substrate inhibitors. Figure 4 also examines fluorescence values by locus with respect to substrate sampling, and further supports the concept that modern extraction methods successfully diminish substrate-based inhibitors from evidentiary samples. The only degradation to sample fluorescence is seen at higher molecular weight targets from the Fusion 6C kit, which is an expected occurrence (Applied Biosystems, 2017; Smith *et al*., 2015).

In conclusion, substrate type does not appear to have a significant effect on forensic DNA analysis based upon the current study results given the concentrations of DNA present on these samples. The capability of these inhibitors to cause major issues with profile development does exist, as observed in the potential diminishment of fluorescent values for leather and denim samples herein and could be heightened with situations involving limited DNA amounts. That being stated, it would appear that automated extraction procedures, alongside careful collection and sampling techniques, can potentially alleviate the burden these inhibitors can present with regards to challenging forensic samples.

Table 1. Sample, Substrate Composition.

Substrate	Body Fluid Composition
Wood	Blood
Leather	Blood
Denim	Blood
Glass (slide)	Blood
Leather	Semen
Denim	Semen
Glass (slide)	Semen
Denim	Blood:Semen
Glass (slide)	Blood:Semen
Leather	Blood:Saliva

Table 2. Substrate and Body Fluid Quantification Values, Single Source Samples.

Quantification Variables:	*Female, Single Source Samples:*				*Male, Single Source Samples:*		
Sample:	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Substrate:	Wood	Leather	Denim	Glass	Leather	Denim	Glass
Body Fluid(s):	Blood	Blood	Blood	Blood	Semen	Semen	Semen
DI Value:	0.820	0.810	0.790	0.920	0.830	0.800	0.780
Total DNA (ng/uL):	3.98	5.60	2.79	6.52	4.81	1.21	2.01
IPC Ct Value:	28.6	28.8	28.6	28.8	28.4	28.3	28.5

Table 3. Substrate and Body Fluid Quantification Values, Two Person (2P) Mixture Samples.

Substrate Quant Values (2P Mixture Items):			
	Sample 8	Sample 9	Sample 10
Substrate:	Glass	Denim	Leather
Body Fluid(s):	Semen:Blood	Semen:Blood	Saliva:Blood
DI Value:	0.810	0.820	0.840
Total DNA (ng/uL):	3.47	1.14	22.5
IPC Ct Value:	28.5	28.1	29.4
Female to Male Ratio:	2.70	2.61	152

Table 4. Examination of Degradation Index Value Variance between Substrate Replicates. Not Applicable (NA) results indicate samples with no replicates.

Substrate:	Denim	Glass	Leather	Wood
Std. Deviation of DI for Substrate Replicates:	0.0153	0.0737	0.0153	NA
Mean of DI for Substrate Replicates:	0.803	0.837	0.827	0.820
Coefficient of Variation:	**0.0190**	**0.0881**	**0.0185**	NA

Table 5. Examination of Variance between Substrate Replicates, DNA Quantity Retrieved. Not Applicable (NA) results indicate samples with no replicates.

Substrate:	Denim	Glass	Leather	Wood
Std. Deviation of DNA Amt. for Substrate Replicates:	0.933	2.30	9.99	NA
Mean of DNA Amt. for Substrate Replicates:	1.71	4.00	11.0	3.98
Coefficient of Variation:	**0.545**	**0.575**	**0.911**	NA

Table 6. Examination of Degradation Index Value Variance between Body Fluid Replicates.

Body Fluid:	Blood	Semen	Semen:Blood
Std. Deviation of DI for Body Fluid Replicates:	0.0580	0.0252	0.00707
Mean of DI for Body Fluid Replicates:	0.835	0.803	0.815
Coefficient of Variation:	**0.0695**	**0.0313**	**0.00868**

Table 7. Examination of Variance between Body Fluids, DNA Quantity Retrieved.

Body Fluid:	Blood	Semen	Semen:Blood
Std. Deviation of DNA Amt. for Body Fluid Replicates:	1.66	1.89	1.65
Mean of DNA Amt. for Body Fluid Replicates:	4.72	2.68	2.31
Coefficient of Variation:	**0.352**	**0.706**	**0.715**

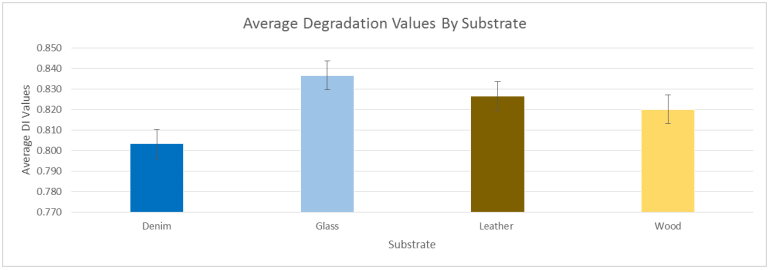

Figure 1. Degradation Values by Substrate.

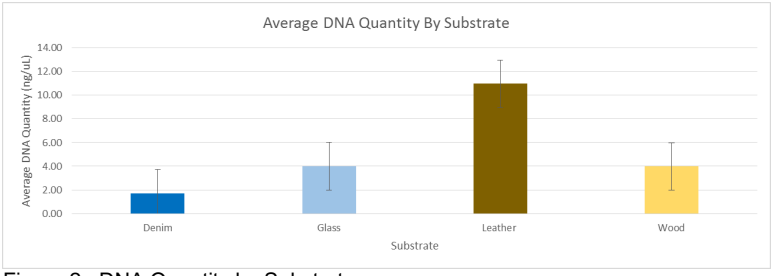

Figure 2. DNA Quantity by Substrate.

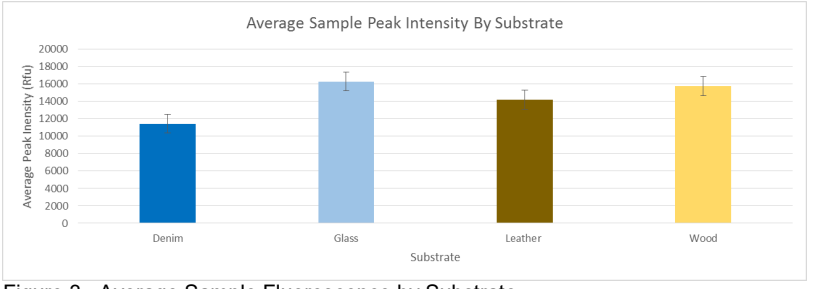

Figure 3. Average Sample Fluorescence by Substrate.

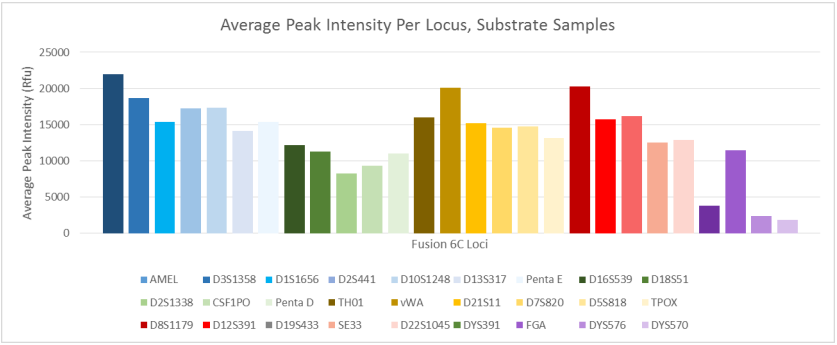

Figure 4. Average Sample Fluorescence by Locus for Substrate Samples.

Minimum Sample Study

Table 1 possesses the major study data, including DNA quantities in each sample and the frequency of artifact and stochastic effect occurrences. All applicable controls for this study passed (data not shown). Large quantities of DNA (4 ng, 2 ng DNA) resulted in electropherogram artifacts (e.g. off-ladder [OL] alleles, pull-up peaks), but full profiles were developed. Full profiles were also generated for all samples above the DNA threshold of 200 pg with little to no artifact development between 1.50 ng to 1.00 ng DNA. This concentration threshold at which stochastic effects (variation in peak heights, allelic/locus dropout resulting from low level DNA amplification) are observed at ≤ 200 pg DNA is similar to what was detected in the Ensenberger *et al*. (2016) developmental validation study performed on the Fusion 6C kit (Perlin, 2010). For the current study, peak height ratio (PHR) imbalance was observed at 400 pg of DNA, but peaks dipping below stochastic threshold (resulting in the potential for sister allele dropout for heterozygous alleles) did not manifest until the 200 pg concentration.

Partial profile development with limited genotype (loci) dropout was observed at a concentration of 60 pg of the diluted DNA. This value is also on par with other validation studies, such as the Cisana et al. validation experiment, where a threshold of 62.5 ng 2800 M DNA resulted in fractional profile generation (Cisana *et al*., 2017). No major profile could be obtained for samples at 20 pg of DNA and below, where most loci had dropped out for such diluted samples.

Table 2 shows the standard deviation, mean, and coefficient of variation values for the replicate samples from the study. As one can determine from the data therein, variation was present between the samples but such variability in fluorescence values should be expected due to sample-to-sample run deviations (e.g. variation in amplification efficiency, capillary separation) and was not substantial. Table 3 examines the PHRs for heterozygous loci from the 2800 M stock DNA profile dilutions. The lower bound or limit of PHRs in this study was examined through the calculation of the mean PHR minus three times the standard deviation for each PHR, which gave a 99.7% confidence interval to the data. As such, the lower PHR limit for samples within the recommended amplification target range of 1.00-2.00 ng of DNA was around 70% and within the limits of published casework test methods (Indiana State Police Section 2.8.12.2, 2017). This finding is very similar to the lower bound PHR values from the Cisana et al. validation study (Cisana *et al*., 2017) which found that a single source profile's heterozygous locus PHR is within 65%. Also of note is that PHR increased with an increased input amount of DNA for these samples. Given that PHR imbalance is a key factor in mixture interpretation and genotype pairing determination, these findings are pertinent from a profile analysis standpoint (Budowle *et al*., 2009).

Figure 1 is a more concise examination of the data from Table 1 and demonstrates the ideal range of DNA input at which the least amount of issues related to artifact manifestation and stochastic effects appears in electropherogram profiles. Per the suggested amplification target range of 1.00-2.00 ng from previously documented methods (Indiana State Police Section 2.8.12.2, 2017), this DNA input range demonstrated minimal artifacts in the electropherograms observed in this study. Furthermore, the samples within this DNA concentration range presented no stochastic effect issues, thereby supporting this range as an ideal concentration series to strive for based on quantification results for a given sample. Figure 2 shows a clear correlation in DNA concentration as it relates to average fluorescent intensity for samples in their respective electropherogram results; while more DNA does lead to larger electropherogram peaks (larger Rfu values from Figure 2), the information should be correlated with Figure 1, where one can

determine the consequences of DNA input on artifact development and stochastic effect appearance in profiles of varying input concentrations. Figure 3 examines mean PHR by amplification input with respect to each locus targeted by the Fusion 6C kit. There was no distinct arrangement in PHR for the heterozygous loci observed in the study samples.

The results of this study are relevant to current forensic DNA applications given how sensitive DNA amplification chemistries have become over time. Multiplex DNA systems have become increasingly popular due to their ability to accurately allow for profile determinations with minimal quantities of DNA (Krenke, 2007). Since the advent of touch DNA analysis around 1997 (Van Oorschot, Ballantyne, and Mitchell, 2010), these sensitive chemistries have become even more important for reliable forensic DNA profile development from small concentrations of DNA and aid in alleviating issues related to DNA mixture assessment. Based upon developmental validation and internal validation studies such as this one, the Fusion 6C system has proven itself to be a potent multiplex system to meet the demands of difficult casework involving mixtures, low template analysis, etc. (Promega, 2015; Patterson, 2015). As issues related to low template profile development are debated (Butler and Hill, 2010; Zimmer, 2016), minimum sample studies will be required to continually validate the ability of multiplex kits to rise to the challenges of forensic sample analysis.

Table 1. Electropherogram Data for Minimum Sample Study.

Study Variables:	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8-1	Sample 8-2	Sample 9-1	Sample 9-2	Sample 10	Sample 11
Total DNA (ng/uL):	0.800	0.400	0.300	0.200	0.120	0.0800	0.0400	0.0200	0.0200	0.0120	0.0120	0.00400	0.00200
Targeted DNA for Amp. (ng)	4.00	2.00	1.50	1.00	0.600	0.400	0.200	0.100	0.100	0.0600	0.0600	0.0200	0.0100
Number Artifact Issues (OL Alleles, Pull-Up):	12	8	5	0	1	0	0	0	0	0	0	0	0
Alleles Below Stochastic Threshold? Yes/No (Y/N):	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y
Number Loci with Observed PHR Imbalance:	0	0	0	0	0	2	2	6	8	8	6	0	1
Number Loci with Allele Dropout:	0	0	0	0	0	0	2	0	0	4	2	2	1
Number Loci Dropout:	0	0	0	0	0	0	0	0	0	2	1	23	24

Table 2. Concordance Data for Study Replicates.

Std. Dev. Average Peak Height Sample 8:	36.0
Mean Average Peak Height Sample 8:	1008
Coefficient of Variation Sample 8:	0.0357
Std. Dev. Average Peak Height Sample 9:	84.6
Mean Average Peak Height Sample 9:	643
Coefficient of Variation Sample 9:	0.132

Table 3. Peak Height Ratio Data for Minimum Sample Study.

Study Variables:	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8-1	Sample 8-2	Sample 9-1	Sample 9-2	Sample 10	Sample 11
Mean PHR Per Sample:	0.937	0.934	0.894	0.889	0.860	0.842	0.804	0.724	0.708	0.663	0.708	NA	0.565
St. Dev. Each Sample	0.0514	0.0627	0.0632	0.0715	0.0877	0.1284	0.1395	0.1861	0.1789	0.1187	0.1691	NA	NA
Lower Average PHR Range:	0.783	0.746	0.704	0.674	0.597	0.457	0.385	0.166	0.171	0.307	0.200	NA	NA

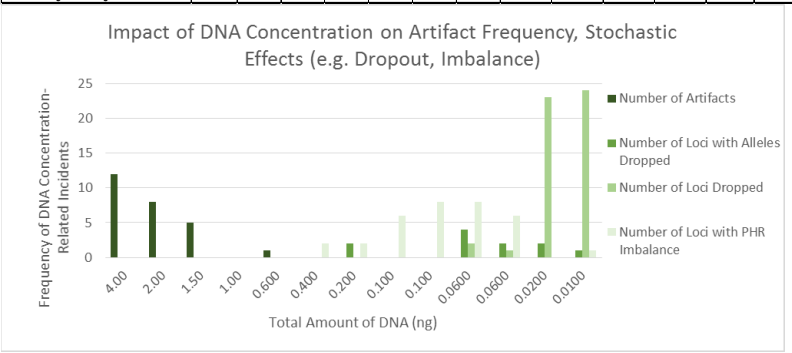

Figure 1. Effect of DNA Input on Artifact and Stochastic Effect Issues.

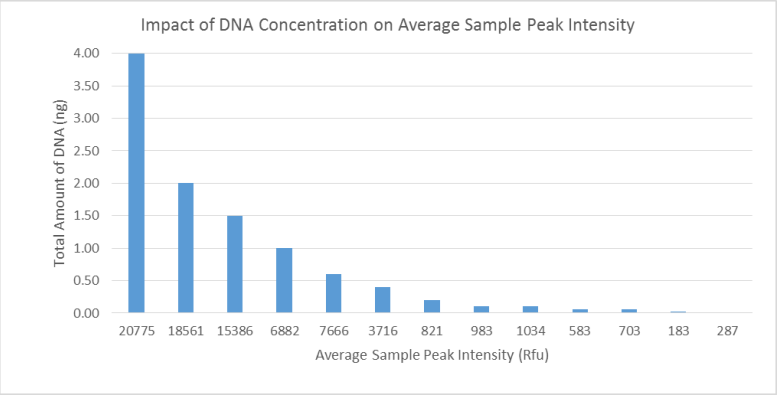

Figure 2. Effect of DNA Input on Average Sample Fluorescence Values.

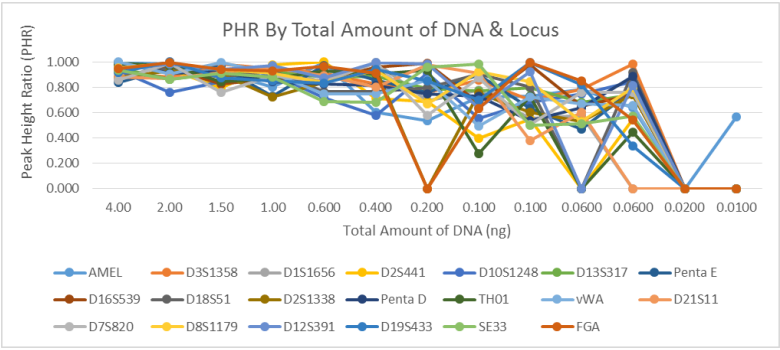

Figure 3. Mean PHR by Locus and DNA Input.

Mixture Sample Study

The examination of mixture samples has become a relatively recent development in forensic DNA analysis. With the advent of novel methods that are sensitive enough to detect only a few molecules of DNA (Zimmer, 2016) and the discovery of touch DNA analysis (Van Oorschot, Ballantyne, and Mitchell, 2010), sample type has drastically swung from predominantly single source DNA profiles to complex and sometimes uninterpretable mixtures of DNA from a variable number of contributors (Bieber *et al.*, 2016). Determining the presence of a mixture is achieved through analyst discretion by way of evaluation of the loci within an electropherogram profile (Grgicak, 2015). An electropherogram profile is "…defined as a mixture of two or more individuals when two or more loci demonstrate additional alleles (i.e. more than two alleles at an autosomal locus or more than one allele at a Y-STR locus)" (Indiana State Police Section 2.8.19.7.4, 2017). Mixture interpretation has made many significant strides recently.

One of the biggest advances in mixture interpretation was the implementation of analytical thresholds and stochastic thresholds in DNA profile analysis to establish increased confidence in making allele calls and determining genotypes for mixture samples (Ryan, 2017). The evaluation of unknown (evidentiary) profiles for mixture genotype contributions prior to known (standard) sample comparison and improved statistical analyses (Random Match Probability, Likelihood Ratio Analysis)

has only further improved the rigor of mixture evaluation in forensics today. SWGDAM has established a series of guidelines for mixture interpretation that compiles best-practice strategies aimed at removing bias in mixture analysis and allowing analysts to achieve the ultimate goal of mixture interpretation—determination of genotype combinations for contributors in a mixture sample (SWGDAM Interpretation Guidelines, 2017). As a result of these endeavors, mixture interpretation today has become common practice to the active forensic DNA analyst.

With respect to this study, Table 3 displays the results of 2P mixture samples. All applicable controls for this study passed (data not shown). Interpretation guidelines were used in accordance with the typical casework test methods for forensic mixture interpretation. The mixture ratio (MR) and the actual MR from combining contributors during sample preparation were accurate from 1:1 to 1:10 samples. As samples approached the 1:20 and 1:50 dilution ranges, greater disparities in MR values were observed.

Major profiles (e.g. single source major profiles with a single source minor profile) were generated for 2P samples for all mixture ratios save for samples where contributor proportions approached 1:1, 1:2 and 1:5 proportions. The 1:1, 1:2, and 1:5 samples resulted in 2P indistinguishable profiles, where the samples did not meet the criteria for pulling a major profile from a sample per the test methods utilized in this study. The inability to determine major profiles from samples with contributor ratios approaching a 1:1 threshold is expected given that the concentration of each contributor from a DNA standpoint is near equal, such that no differentiation from contributors may be discerned. When samples approached ≥ 3:1 average mixture ratio (AMR), 12 or more autosomal loci can be pulled from the mixture sample, allowing for a single source major profile to be generated (Indiana State Police Appendix 2, 2017). While there are no constraints on minor loci to be pulled from a minor profile generated in a single source major with a single source minor profile scenario, less information is gleaned from the minor profile (e.g. fewer alleles pulled) as the contributor ratio approaches 1:20 and 1:50. As Figure 1 illustrates, the best

differentiation and development of major and minor profile loci occurs around the 1:5-1:20 mixture ranges.

Table 4 displays the results from the 3P mixture samples. All three samples would be rendered as indistinguishable 3P mixtures. The rationale for the decision to classify the three, 3P mixture samples as indistinguishable arises from the fact that the samples failed to meet the requirements for a single source major profile and a two person mixed major profile stipulated methods manual used for this study. That stated, all major profiles were correctly displayed in spite of samples failing to meet the requirements for alternative classifications.

In conclusion, the study results for mixture interpretation and analysis continue to support the notion that 2P mixture samples can still be reliably analyzed in most situations. With respect to 2P mixture sample results, the study demonstrated successful amplification of samples up to a 1:20 mixture ratio, where samples were classified as either indistinguishable mixtures or single source major with a single source minor profile. Sample minor loci information began to decrease in samples with ratios of 1:20-1:50 in this study. In regards to the 3P mixture portion of this study, all three samples were classified as 3P indistinguishable mixtures per the test methods. That being stated, 3P mixtures should be examined with caution given the challenges associated with distinguishing between the different classifications of 3P mixtures.

Table 1. Two Person Mixture Sample Setup.

Mixture Ratio (Female:Male) or (Male:Female)	Amount Contributor 1 (Amount DNA ng/uL)	Amount Contributor 2 (Amount DNA ng/uL)	Total amount DNA in dilution (Amount DNA ng/uL)
1:1 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	5 uL Dilution 6 (0.3 ng/uL)	10 uL, 3 ng total DNA (0.3 ng/uL solution)
1:2 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	10 uL Dilution 6 (0.3 ng/uL)	15 uL, 4.5 ng total DNA (0.3 ng/uL solution)
1:5 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	25 uL Dilution 6 (0.3 ng/uL)	30 uL, 9 ng total DNA (0.3 ng/uL solution)
1:10 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	50 uL Dilution 6 (0.3 ng/uL)	55 uL, 16.5 ng total DNA (0.3 ng/uL solution)
1:10 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	50 uL Dilution 6 (0.3 ng/uL)	55 uL, 16.5 ng total DNA (0.3 ng/uL solution)
1:20 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	100 uL Dilution 6 (0.3 ng/uL)	105 uL, 31.5 ng total DNA (0.3 ng/uL solution)
1:50 (M:F)	5 uL Dilution 4 (0.3 ng/uL)	250 uL Dilution 6 (0.3 ng/uL)	255 uL, 76.5 ng total DNA (0.3 ng/uL solution)
1:1 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	5 uL Dilution 4 (0.3 ng/uL)	10 uL, 3 ng total DNA (0.3 ng/uL solution)
1:2 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	10 uL Dilution 4 (0.3 ng/uL)	15 uL, 4.5 ng total DNA (0.3 ng/uL solution)
1:5 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	25 uL Dilution 4 (0.3 ng/uL)	30 uL, 9 ng total DNA (0.3 ng/uL solution)
1:10 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	50 uL Dilution 4 (0.3 ng/uL)	55 uL, 16.5 ng total DNA (0.3 ng/uL solution)
1:10 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	50 uL Dilution 4 (0.3 ng/uL)	55 uL, 16.5 ng total DNA (0.3 ng/uL solution)
1:20 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	100 uL Dilution 4 (0.3 ng/uL)	105 uL, 31.5 ng total DNA (0.3 ng/uL solution)
1:50 (F:M)	5 uL Dilution 6 (0.3 ng/uL)	250 uL Dilution 4 (0.3 ng/uL)	255 uL, 76.5 ng total DNA (0.3 ng/uL solution)

Table 2. Three Person Mixture Sample Setup.

Mixture Ratio	Amount Contributor 1 (Amount DNA ng/uL)	Amount Contributor 2 (Amount DNA ng/uL)	Amount Contributor 3 (Amount DNA ng/uL)	Total amount DNA in dilution (Amount DNA ng/uL)
3:1:1 (F:M:M)	15 uL Dilution 6 (0.3 ng/uL)	5 uL Dilution 1 (0.3 ng/uL)	5 uL Dilution 4 (0.3 ng/uL)	25 uL, 7.5 ng total DNA (0.3 ng/uL solution)
3:3:1 (M:M:F)	15 uL Dilution 1 (0.3 ng/uL)	15 uL Dilution 4 (0.3 ng/uL)	5 uL Dilution 6 (0.3 ng/uL)	35 uL, 10.5 ng total DNA (0.5 ng/uL solution)
1:1:1 (M:M:F)	5 uL Dilution 1 (0.3 ng/uL)	5 uL Dilution 4 (0.3 ng/uL)	5 uL Dilution 6 (0.3 ng/uL)	15 uL, 4.5 ng total DNA (0.5 ng/uL solution)

Table 3. Two Person Mixture Interpretation Results.

	Prepared Ratio	IndySTR MR	Number Major Loci	Number Minor Loci	Indistinguishable?	Major/Minor?
Sample 1	1:1 (M:F)	1.90	5	1	x	
Sample 2	1:2 (M:F)	3.32	14	10		x
Sample 3	1:5 (M:F)	9.12	23	19		x
Sample 4	1:10 (M:F)	12.3	23	18		x
Sample 5	1:10 (M:F)	13.5	23	17		x
Sample 6	1:20 (M:F)	24.4	23	16		x
Sample 7	1:50 (M:F)	30.4	23	7		x
Sample 8	1:1 (F:M)	1.80	6	3	x	
Sample 9	1:2 (F:M)	1.41	2	0	x	
Sample 10	1:5 (F:M)	2.68	10	6	x	
Sample 11	1:10 (F:M)	4.60	14	11		x
Sample 12	1:10 (F:M)	4.47	14	10		x
Sample 13	1:20 (F:M)	8.84	20	17		x
Sample 14	1:50 (F:M)	13.0	23	11		x

Table 4. Three Person Mixture Interpretation Results.

Sample	Prepared Ratio	Max Alleles Detected at Locus	Number Loci Pulled for Major Profile
Sample 15	3:1:1 (F:M:M)	6	2
Sample 16	3:3:1 (M:M:F)	6	1
Sample 17	1:1:1 (M:M:F)	6	1

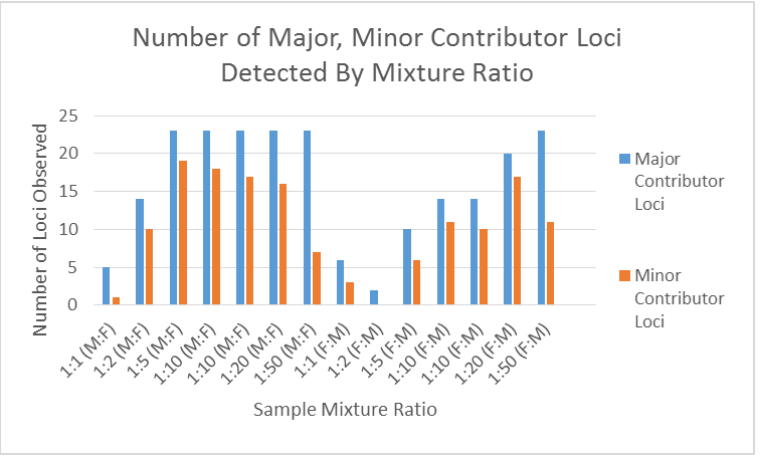

Figure 1. Major and Minor Loci Detected for Two Person Mixture Ratio Samples.

Casework Study

Casework sample data can be observed in Table 1. All applicable controls for this study passed (data not shown). Of the eleven samples processed, only one sample (anal swab sperm fraction [SF]) fell below a quantification value of 0.01 ng/uL DNA. The small autosomal 0.01 ng/uL DNA value has been determined to be the minimum amount of DNA needed to proceed with autosomal STR analysis (Schienman, n.d.). As expected with this sample, no DNA profile was generated as a result of insufficient DNA being present in this sample. All samples with DNA above the 0.01 ng/uL limit were successful in the amplification and development of either single source or mixture profiles for interpretation.

The average standard IPC Ct value for the casework samples quantified was 28.6. Eight of the casework samples processed had IPC Ct values below the average standard IPC Ct, indicating that there was no inhibition. Three samples (internal genital swab non-sperm faction [NSF], external genital swab NSF, and vaginal/cervical NSF) had IPC Ct values higher than the average standard IPC Ct value, indicating that some inhibition was occurring during quantification. These three samples had large amounts of DNA present in their extracts, which is the likely variable that led to higher IPC Ct values for

these items. As examined in earlier study segments, the DI value is used to assess sample quality in terms of template degradation. All samples from the study fell within the slight to moderate degradation range and presented no substantial contrast between sample type and extraction type. The female to male ratio is used to calculate the amount of autosomal DNA present in a sample in proportion to male DNA based on the Y-chromosome target found in the Quantifiler Trio kit (Applied Biosystems, 2017). Given that an increase in the female to male DNA ratio can result in restricted minor male profile information from a sample, one can determine that the higher ratios presented in Table 1 correlate to the types of profiles visualized in the electropherograms developed from the samples in this study.

In regards to profile interpretation, most samples gave a single source profile. These samples were single source, female profiles that represent the victim profile given the samples were of an intimate nature. One sample provided a 2P mixture (dried secretion swab) and upon deducing from the intimate single source sample profile from the victim, a single source major male profile was obtained alongside the female profile as a minor contributor for the sample. There was also one sample (external genital swab SF) that resulted in an indistinguishable 2P mixture. The results of this study indicate that the Maxwell extraction protocol, combined with Quantifiler® Trio quantification and Promega® PowerPlex® Fusion 6C amplification chemistries are sufficient for informative result development with respect to casework samples; the amplification success echoes observed results found in the Fusion 6C developmental validation publication (Ensenberger *et al*., 2016). In spite of varying quantities of DNA from an assortment of samples, the chemistries used to process forensic biology casework for DNA profile development were successful at generating various types of profiles (e.g. single source, 2P mixtures) for evaluation. The minimal 0.01 ng/uL DNA limit for autosomal STR analysis remains to be a reliable guidepost for profile development as observed in the previous validation study (Cisana *et al*., 2017). DI and IPC Ct were demonstrated to be sufficient quality indicators with regards to sample integrity (Applied Biosystems, 2017). These results, therefore, corroborate the literature

(Schienman, n.d.; Bader, 2016) indicating that the Fusion 6C chemistry, alongside the aforementioned extraction and quantification chemistries in place, are reliable systems for processing casework samples and in particular appear to meet the challenges of ever-increasingly difficult casework samples such as those with low template, degradation, and mixture-based obstacles to successful data acquisition.

Table 1. Casework Study Sample Results.

Sample	Description	DNA Amt. (ng/uL)	IPC Ct	DI	F:M Ratio	Total Amt. DNA Amp. (ng)	Result
1	Scraping Swab (Left)	0.0231	28.3	2.03	18.3	0.347	Single Source Profile
2	Scraping Swab (Right)	0.0129	28.3	1.17	17.4	0.194	Single Source Profile
3	Dried Secretion Swab ("kissed, licked")	0.769	28.4	1.09	<1	1.50	Major/Minor Profiles
4	Anal Swab NSF	0.0279	28.1	3.24	278	0.419	Single Source Profile
5	Anal Swab SF	0.000400	28.2	4.00	NA	0.006	No Result
6	External Genital Swabs NSF	17.0	29.6	2.23	>999:1	1.50	Single Source Profile
7	External Genital Swabs SF	0.0246	28.2	2.02	10.7	0.369	Indistinguishable
8	Internal Genital Swabs NSF	27.9	30	1.35	683	1.50	Single Source Profile
9	Internal Genital Swabs SF	20.6	28.2	1.64	171	1.50	Single Source Profile
10	Vaginal/Cervical Swabs NSF	31.5	29.1	2.12	>999:1	1.50	Single Source Profile
11	Vaginal/Cervical Swabs SF	0.047	27.9	2.03	41.5	0.702	Single Source Profile

Precision Study

Table 1 displays the values from the precision study. All applicable controls for this study passed (data not shown). The 16 samples analyzed resulted in a total of 6,912 called alleles. All replicate sample alleles were sized and typed properly based upon the allelic ladder composition. No recorded alleles across all loci examined resulted in a standard deviation from the mean allele size (data not shown) greater than 0.15 base pair (bp), with a maximum standard deviation of 0.100 (Penta E, allele 11) and a minimum standard deviation of 0.0271 bp (D21S11, allele 33.2). Moreover, the average standard deviation for each locus was below 0.15 bp, with a maximum average standard deviation of a locus at 0.0821 bp (Penta E) and a minimum average standard deviation of a locus at 0.0460 bp (D2S441). Precision for the 3500 genetic analyzer (or any measurement system for that matter), which separates and detects DNA fragments, has to be below ± 0.5 bp to accurately differentiate between partial repeat alleles (microvariants) and true 1 bp allele complete repeats for a given locus (Butler, 2005). As such, all samples analyzed for this study demonstrated precision within the limits stipulated in the literature. These values reflect consistent precision with the Fusion 6C system akin to results also demonstrated in similar validation studies using this system (Jiang, n.d.). Given how important sizing precision is to accurate

genotypic determinations (Ensenberger, 2016), the following study has demonstrated the precision of the Fusion 6C system with regards to accurate allele labeling.

Table 1. Precision Study Data for Allelic Ladder Samples.

Locus	Average Std. Dev. Per Locus	Max. Std. Dev. At Locus	Min. Std. Dev. At Locus
AMEL	0.0723	0.0758	0.0688
D3S1358	0.0514	0.0671	0.0395
D1S1656	0.0549	0.0692	0.0433
D2S441	0.0460	0.0575	0.0328
D10S1248	0.0765	0.0907	0.0601
D13S317	0.0707	0.0893	0.0571
Penta E	0.0821	0.100	0.0677
D16S539	0.0538	0.0721	0.0335
D18S51	0.0510	0.0926	0.0279
D2S1338	0.0541	0.0541	0.0403
CSF1PO	0.0674	0.0749	0.0584
Penta D	0.0537	0.0805	0.0344
TH01	0.0679	0.0914	0.0565
vWA	0.0475	0.0594	0.0346
D21S11	0.0487	0.0645	0.0271
D7S820	0.0604	0.0713	0.0485
D5S818	0.0511	0.0748	0.0344
TPOX	0.0705	0.0937	0.0465
D8S1179	0.0598	0.0844	0.0446
D12S391	0.0500	0.0627	0.0405
D19S433	0.0543	0.0694	0.038
SE33	0.0568	0.0752	0.0344
D22S1045	0.0478	0.0657	0.0336
DYS391	0.0601	0.0711	0.0543
FGA	0.0606	0.0903	0.0385
DYS576	0.0596	0.0706	0.0461
DYS570	0.0529	0.0724	0.0367

Non-Human (Species Specificity) Study:

With forensic PCR kits using smaller repeat nucleotide targets across several loci, and given that the molecular composition of DNA is limited to a mere four-letter series of bases arranged in various combinations, the concern for possible cross-reactivity in newly selected targets and analytical chemistries is valid. Being able to target species-specific transcripts, such as human DNA in forensic biology casework, is

important so as to not waste samples of a limited size, minimize reagent waste, and ensure accurate profile development (Solt and Kanthaswamy, 2014). Because there is a high likelihood that traces of non-human DNA (e.g. animal, bacterial sources) may be found on evidentiary items being processed for human DNA profile development, and because the fact exists that primers used in amplicon development can identify with non-human binding sites from these fragments of non-human DNA, species specificity studies are thoroughly conducted during developmental validation of a given PCR chemistry (Ensenberger, 2016). The ultimate goal of such studies (Berbisin *et al*., n.d.; Green *et al*., 2005; Barnard Health Care, 2015) is to ensure that cross reactivity is a non-issue with respect to amplification, ensuring that only those targets and biological systems associated with those targets central to a PCR chemistry are detected when present from a given sample.

The results from Quantifiler Trio quantification (data not shown) resulted in no DNA being detected for the non-human samples. All applicable controls for this study passed (data not shown). There was no DNA detected or quantified for the Quantifiler Trio chemistry and its small autosomal, large autosomal, and Y chromosome DNA targets. In spite of lacking the minimum amount of small autosomal DNA for Fusion 6C amplification, 0.01 ng/uL DNA (Indiana State Police Section 2.8.11.7.4.1, 2017), an attempt to develop an electropherogram profile for all non-human samples was performed to confirm the specificity of the amplification kit alongside that of the quantification chemistry.

With regards to Fusion 6C amplification (data not shown), no profile peaks were detected for any of the non-human samples. No artifacts or peaks above the analytical threshold were present in the electropherograms that were described in the Fusion 6C technical manual (Promega, 2017) for the non-human samples in this study. The results of this study closely parallel the results observed from the Fusion 6C developmental validation (Ensenberger *et al*., 2016), thereby demonstrating reliable human-centric amplification of the targets carefully selected for this kit. To conclude, the results of this part of the study indicate that the primers designed for the Fusion 6C kit, much akin to

observations regarding the Quantifiler Trio kit, are highly specific with regards to human-based target amplification.

Concluding Remarks

The goal of this experiment was to validate the performance capabilities of the systems and chemistries employed by in routine forensic biology casework analysis. The Applied Biosystems® Quantifiler Trio DNA Quantification Kit and the Promega® PowerPlex Fusion 6C System and all applicable equipment and software were challenged with a unique range of sample types to mimic the wide variety of samples that may present themselves in a forensic casework setting. A diverse array of samples tested the limits of these methods: environmental insults, varying substrates, limited DNA template, DNA mixtures, non-probative casework items, non-human blood sources, and replicate precision samples. In spite of all these challenges, the chemistries and equipment utilized performed in a robust fashion with reliable results that align with previous validation study outcomes across parallel studies of a similar nature. In summation, the genotyping systems and applicable chemistries confronted through this validation study continue to remain dependable processes for forensic casework examination and prove that forensic DNA analysis truly remains one of the most well-studied and scientifically rigorous disciplines within the realm of forensic science.

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Simulated Stress: An Analysis of Video Game Genres in Relation to Possible Stress Reactions in Players from a Neurological Perspective

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Abstract
This essay offers an analysis of video game genres in relation to stress reactions in players who already have a higher level of stress than average. After a literature review summarizing current research on stress and prior studies related to stress and gaming, the analysis of the game genres will suggest several major points in regards to stress. The genres under analysis as potentially stress decreasing are city builders, 4 X strategy, and puzzle games. Genres which could possibly increase stress levels are first person shooters, real time strategy games, and the survival horror genre. Of note in the analysis is the suggestion that the way in which varying game genres deal with in-game time in relation to real world time has an impact on players stress levels. In short, the more often a game genre requires faster reflexes and reaction times, the higher the likelihood of increased stress in players. Further points in the analysis propose that players may not be aware of a game increasing their stress level because though they are becoming more stressed, they are simultaneously enjoying themselves. A final section as part of the conclusion suggests implications for future research can be found in future studies which tests these possible connections using qualitative and/or quantitative research.

Introduction

The player leans forward. Eyes focus on screen. A sheen of perspiration glistens on her brow. Fifteen hours for this moment.

The player takes a slow, deep breath, trying to calm down. Then, in the blink of an eye, it's over. A mistake she didn't even know she was making infuriates her as the words "You Died" fade in on screen. Her jaw tightens and she grips the controller. Suddenly, the room begins to spin. The kick of adrenaline isn't wearing off as it should, but only increasing. She breaths faster; her heart pounds out of control.

Over the last several decades, stress and anxiety have become an increasing part of our culture. While the reasons for this increase are under debate, it would be difficult to argue that the population's ever-growing interconnectedness through electronic media had no impact on the number of those affected. Video games in particular have been studied for their effect on human mental processing in a number of contexts. Although no direct cause and effect relationship has been determined between gaming stimuli and stress, recent studies in how games can affect stress levels have begun to shed new light on the subject. However, though particular games have been analyzed in the context of stressful gameplay, stress in players has not been directly addressed in the context of the mechanics that constitute video game genres. This being the case, an analysis of varying video game genres will suggest that for those struggling with high levels of stress, certain genres of video games could potentially work well as a coping mechanism while others may exacerbate symptoms. Moreover, the way in which game genres deal with time is an integral factor in assessing the potential for inducing stress. The benefit of this analysis is in creating a better understanding of what design factors may affect players in relation to stress, and thereby determine the actual impact of game genres on those suffering with high levels of stress. This could then be better understood and measured through future qualitative and quantitative research, as well as information that could potentially aid those affected by high stress levels.

Before conducting this discussion, it is important to have a clear definition of terms to work with. Mental disorder classifications such as stress, anxiety disorder, and other terms are often ambiguous, with each individual person being their own unique case. Thus, the literature review will first summarize stress and anxiety disorder from neurological, emotional, and

social perspectives. Following this will be a look at gaming studies and how they can alter mood and stress levels in both positive and negative ways.

Stress and Anxiety

In the common vernacular, terms like stress, anxiety, and worry are often used interchangeably. Indeed, stress is a nearly universal trait among humans. As such, this study will not focus on those who experience stress in reasonably stressful situations. For example, if someone was nearly in a car wreck, then a stress response would be reasonable. However, with the increasingly common occurrence of generalized anxiety disorder (GAD) and similar disorders due to the over response of the autonomic nervous system, the distinctions between day to day stress and debilitating anxiety becomes relevant. In looking at the current state of psychological research, it becomes clear that stress related issues come in varying degrees and have a wide array of psychological and physiological symptoms.

In very broad terms, those suffering from diagnosable forms of stress related disorders fall into two general categories. First, those with GAD suffer from a “chronic condition that involves pervasive, excessive, and uncontrollable worry about multiple sources of potential threat” (Kircanski et. al. 2016, 320). While certain events such as physical or emotional pain, divorce, or unemployment can be predictors of GAD, the disorder itself is defined by the obsessive nature of mental processing about the perceived threats to the individual (Watterson et. al. 2017). The other major categorization of interest to the current study is panic disorder (PD). This can be defined as “a dramatic anxiety syndrome characterized by recurrent episodes of acute fear” which “encompasses aspects of acute fear (spontaneous and cued panics), chronic anxiety (anticipatory fear), [and] interceptive sensation sensitivity” (Goddard 2017, 1). While the direct cause of PD is unclear, some recent research suggests it could be tied to factors such as genetics, temperament, and potentially chronic stress (Goddard 1). What is of note here is that GAD itself does not fit the stressful experiences found in some game genres. Indeed, games rarely induce “uncontrollable worry.” However, a few traits of GAD do appear in tangential relation to gaming insomuch as it can be connected to PD. In

summation, both GAD and PD share many similarities, and indeed, severe GAD is often associated with PD.

It is also important to note that in this study when high stress levels are discussed, they are meant less in the clinical sense, but rather as a way of implying an inclination towards some of the traits described here. Clinical diagnoses of GAD and PD remain a non-exact science, and as such the genre analysis will assume some level of symptoms of these disorders rather than a confirmed diagnosis. In looking at how video games could potentially impact those suffering from these disorders, it is important to first begin to understand the brain functions that can lead to some of these negative outcomes.

Neurological Perspective

In a discussion of how game genres can affect the ways in which our brain processes stress, it is imperative to begin with a basic understanding of how the brain copes with stressors and what is going wrong in those suffering from heightened anxiety. While the study of anxiety and panic disorders traces a history back to Freud and beyond, current research is still hypothesizing as to the neurological aspects of the disorders. Turning first to GAD, Kircanski's (2016) study focused on how a GAD group and control group remembered day to day situations from their lives and what types of information they recalled. Ultimately the study concluded that those suffering from GAD exhibited "impaired inhibitory control over threat information" (Kircanski 324). In other words, those suffering from GAD were able to process information that could potentially be a threat as normal, but were not able stop thinking about that information once the threatening situation had passed. To return to the driving illustration, suppose someone with GAD accidently cut off another person in traffic and nearly caused an accident. Where a person without GAD might react with fear in a reasonable way during the incident and immediately thereafter, someone with GAD would continue to dwell on the near-collision for a significant time afterwards. Moreover, they would imagine scenarios wherein they might be arrested for bad driving or hunted down by the other driver with a case of road rage.

In looking at the neurological aspects of PD, Goddard explains that past research has suggested "brainstem structures

mediated acute fear expressions (panic), while chronic anticipatory anxiety was mediated via the limbic structures and phobic avoidance was processed via the [cortical structures]" (Goddard 6). In other words, these three types of fear response which could widely be categorized as stress, panic, and a phobia, took place in separate parts of the brain. What is of particular note to this study however is that "the thalamus, an important sensory integration node, has been found to be hyperactive across a range of anxiety disorders in activation and function imaging studies" (Goddard 6). Further, it is suggested that "poor sensory inhibition (to internal and external stimuli) in PD" is common. Goddard then argues that "there is emerging evidence of difficulties with sensory processing in several modalities in PD that could bias patients towards threat sensitivity and fearful responding" (Goddard 6).

As it relates to the study at hand, Goddard is suggesting that those with PD have difficulty processing sensory information and determining on a neurological level what sensory input – sight, sound or otherwise – could potentially indicate a threat. It is important to note here that these discussions are not factoring in the rational part of the brain, but rather highlighting that PD bypasses that type of mental processing altogether. Moreover, this trait of difficulty in threat assessment relates to Kircanski's description of GAD.

Along these same lines, another study by Lieberman et. al. (2017) looks into how uncertainty factors into PD. The authors note that "among individuals who have experienced a panic attack, greater self-reported intolerance of uncertainly is positively associated with the severity of PD symptoms" (Lieberman et. al. 51). What is of particular import in relation to our current study however is Lieberman's focus on how individuals with PD cope with unanticipated threats (U-threat) in the form of the startle response.

After testing PD sufferers as well as a control group with a NPU-threat startle test (Schmitz & Grillon 2012), the study found that "converging lines of evidence indicate that exaggerated anticipatory anxiety in response to U-threat is a core dysfunction in PD" (Lieberman et. al. 59). Indeed, the level and severity of panic symptoms in those with PD when faced with being startled was much higher than others. Considering the aversion that

most suffering from PD have to being surprised, this does stand to reason. One interesting argument made in this work is that how those suffering from PD score on the startle test may be a good treatment target for assessing progress in dealing with the disorder. Both GAD and PD are rooted in the brain, but it would be remiss to ignore social and emotional aspects as contributors to these conditions.

Social and Emotional Perspective

GAD and PD, much like other forms of psychological disorders, can carry with them a negative social stigma. A recent (2017) Canadian study suggested that individuals with GAD experience significant pain, stress, stigma, and discrimination (Watterson et. al. 28). Along with this broader sociological context, these disorders also take place in the emotional context of day to day life. A work by Marsh and De Los Reyes (2018) suggests that someone generally doesn't express that they have been "feeling anxious lately; rather, she expresses this mental health concern alongside talking about stress at work, issues with a family member, and concern over the current political climate" (189). Indeed, this aspect of PD is relevant insomuch as it highlights the broader contextual causes of PD and GAD.

Another important finding in the Marsh and De Los Reyes study related to the argument is that "panic disorder symptoms were interpreted differently depending on the context in which they present" (198). Further, they found "evidence that even when a mental health disorder is not characterized by symptoms that are tied to the context in which a patent presents, contextual information still exerts an influence on judgments about those symptoms" (198). In the case of this study, what is of note is this notion of context. Marsh and De Los Reyes are suggesting that "mental health symptoms are not readily separable from the context in which they present" (189). The relevance here is that to a gamer, the gaming experience itself is that context.

While progress has been made in understanding the biology and symptoms, the causes of PD and GAD are extremely varied. Why exactly a particular situation can cause panic while another has no effect is only beginning to be understood. However, in this short survey of the literature it could be argued that the factors of unexpected events and sensual stimulation are

perhaps some of the more common factors in these disorders. This being the case, a look at the ways in which gaming impacts the brain will aid in seeing how gaming can relate to PD and GAD.

Gaming and the Brain

With this understanding of the ways in which the brain can respond in stressful situations, it begins to become clear the ways in which gaming could impact brain chemistry. Moreover, when brain chemistry is affected, there is a demonstrable impact on real world behavior. The effect of gaming on behavior is a well-studied topic, and indeed, far too large to address completely in the context of this work. However, before focusing on how differing genres of games alter the brain, we must understand that differing genres of games work at fundamentally different levels. In much the same way that taking a stroll in the park and drag racing are both modes of locomotion, so the genres of video games available are so varied that the term 'game' hardly encompasses the range of experiences. This then leads us to a discussion of how these varying genres impact the way our brains work.

A 2013 study conducted by Schmidt et. al. directly addresses the issue of how simulations can impact real life behavior. They first argue that prior research supports the idea that players of video games have improved "cognition, spatial and situation awareness, reaction time, as well as other performance measures" (1450). This in turn leads to the primary hypothesis of the study, namely, that gamers of action video games will more effectively cope with stressful situations and more easily improve their performance upon critique than non-gamers. In this context, Schmidt et. al. defined "action video games" as games "consisting of physical and temporal challenges involving hand-eye coordination movements through controllers or motion/gesture based systems,…[that] involve reasoning and opportunities for character growth, and provide goals a player can work towards achieving" (1450). The experiment consisted of having both gamer and non-gamer participants patrol a recreated Afghani village for improvised explosive devices. The study showed that players of action video games did indeed have better response times, fewer false

alarms, and higher correct detections of bombs. Moreover, the gamers reported that they perceived the workload of the tasks as less burdensome. What stands out, however, in the context of the current study is that despite being statistically insignificant, worry levels tended to be higher in participants who were gamers. As the study notes, "even though not a significant finding for this study, [gamers] were found to have higher worry scores than their [non-gamer] counterparts" (1453). This, then, suggests that though there were positive effects of gaming on the ability of players, there were also potential negative aspects on an emotional level. Fischer et. al. (2009) also offers support for the notion that video games can on some level impact behavior. Through a series of four studies on the effect of driving simulation games on players, they found overall that "racing games increased risk taking" (1407).

From a physiological perspective, West, Konishi, and Bohbot argue that games can directly impact brain chemistry. Their article takes an approach based in prior work on hippocampus-dependent spatial learning, relating it to various aspects of game design. For the most part, their work pertains to the ways in which video games depict space and how our brains comprehend and learn from that movement. The authors make a distinction between *response learning*, which relates to reward-punishment along with memorization, and *spatial learning* which is "thought to be driven by curiosity and novelty detection" (West, et. al. 154).

What is of particular note here is their hypothesis that certain design attributes directly relate to the two types of learning. For example, slower paced games with more novel environments that encourage exploration favor Hippocampus-dependent spatial learning. However, games which encourage in-game GPS, are faster paced, and have high levels of repetition with little novelty encourage non-hippocampal response learning. While these arguments are not directly tied to the notion that particular games can cause or reduce anxiety, it does overlap the idea that game design can have an impact on the function of the brain.

Aggression

The link between brain chemistry and real-world behavior however is not always quite so clear. A 2017 study conducted by Ferguson et. al. found that contrary to their original hypothesis, "violent content in video games had no discernable impact on behavioral mood or outcomes at all" (p. 334). They go on to acknowledge that in light of other recent research which also supports their findings, the previously supported notion that exposure to violence in games can increase aggression in players may in fact be "a part of the larger replication crisis influencing psychological science" (p. 335). That is to say, studies that showed a connection between violence and video games benefited from publication bias due to the zeitgeist of the early 2000s in which scholars presumed the connection existed. However, the literature search of Greitemeyer and Mugge (2014) of the impact of gaming on behavior includes 98 different studies with a total of 36,965 participants and "reveals a positive link between video game play and social outcomes for experimental, correlational, and longitudinal studies" (p. 585). Moreover, they also take direct issue with Fergusons prior works, suggesting that "publication bias did not account for the effect [found in the meta-analysis], which also strongly contradicts Ferguson's claims" (p. 584). Clearly the topic remains a polarizing issue among scholars.

In regards to the current study, this information has been included due to the inevitable link made between brain chemistry and behavior. Indeed, brain chemistry is at the root of behavior, but alterations in brain chemistry can and often do take place independently of social behavior. Moreover, anxiety disorder is just that – a disorder – not a behavior. While, according to Greitemeyer, the Ferguson study did not provide evidence to support contentions that exposure to violent video games contributes to heightened aggression, stress or hostility, their sample did not select individuals who already had a brain chemistry related primed for aggression (p. 584). Further, this study does not attempt to make the argument that games can cause stress in a healthy individual, but rather the notion that they can exacerbate a pre-existing inclination towards stress reactions.

A related 2012 study conducted by Whitaker and Bushman approached gaming from an alternate perspective – in particular, the effect of relaxing video games. The authors in fact suggest a similar idea to the current study, stating that "video games may be the ideal form of media for managing mood states because they are active rather than passive" (88). They go on to note that "just as playing violent games can induce negative feelings, playing relaxing games can induce positive feelings" (89). In their experiments, participants played *Endless Ocean* and the fishing mini-game from *The Legend of Zelda: Twilight Princess* as examples of relaxing games. Results indicated that after playing a relaxing game, participants showed decreased aggression and more kindness than other participants who had been playing violent games.

Stress

Along the same lines, and indeed in a similar idea to the current study, a 2014 study from Bouchard et. al. "asserted whether immersive simulation in a FPS/horror 3D game could induce sufficient stress in military personnel in order to, in a later study, implement stress-management training" (348). The study found some interesting and at times seemingly contradictory information. For example, while the study did find a statistically significant number of participants experienced an increase in both heart rate and breathing congruous with a control stress test, several participants breathing rate increased while their heart rate remained stable (339, 350). This could partially be explained by the fact that the subjects were military personnel and had therefore been trained in firefight situations, thus decreasing their autonomic response to a simulated battle situation. Whatever the case, this does support the notion that gaming can induce a physiological stress-response in player.

Prior research also addresses the issue as to whether games can reduce stress in players. A 2017 work by Rupp et. al. suggests that games indeed can reduce stress levels. The authors looked at how casual gaming impacted work related stress and fatigue levels, particularly in those with critical high-stress jobs such as security or medical professions. The study concluded "that playing casual video games could be an effective and even fun way of recovering from such fatigue, at

least in the affective domain" (1105). What is of particular note here is the authors acknowledgment that "decreasing stress, increasing mood, and having fun are not completely interchangeable" (1105). In the Rupp et. al. study, participants played the game Sushi Cat 2, a "pachinko-type game…prototypical [of] casual games" (1099). That is to say, it was not a game that involved a significant amount of mental rigor on the part of the player. This being the case, the study is not reflective of all types of games. Indeed, Rupp et. al. notes, "players state that they are having fun even when playing video games that are stressful" (1106). Stress, then, is not a significant enough factor to turn players away from certain games, which in turn leads to one of the main ideas in the current study. Namely, that a player can be enjoying themselves while at the same time exacerbating their stress levels. This then prompts the question of how one can distinguish between games that can reduce anxious feelings and games that can induce them. Ultimately, as we argue, this comes down to a question of genre.

Game Genres

In looking at the ways in which gaming can help or hinder individuals with anxiety disorder, it is important to attempt to determine exactly which design elements can lead players to increased stress. Based on the literature review, stress can be tied to a number of factors. Violence in games, the level of dexterity and reaction time required to succeed, time sensitive game mechanics, and elements included to intentionally frighten players such as in horror games are all design elements which are often tied to specific genres. Other genres, however, avoid these elements entirely and present the player with challenges rooted more in critical thinking, creative problem solving, and peaceful interaction. What follows is an overview of three popular genres which could potentially aid in dealing with anxiety, and three genres which could have negative effects on those already suffering from anxiety disorder. That said, Clarke (2017) argues, "Video games are interactive processes. Yet contemporary video game genre labels and classification systems fail to capture the novel complexity inherent in these games" (446). Thus, while this study will use the term 'genre' in a broad sense, the main focus

will be on how the aforementioned traits within game mechanics can interact with anxiety disorder.

Genres That Help

As noted, many games contain elements which this paper will argue can be helpful to those suffering from anxiety disorder. Critical thinking, creative problem solving, and peaceful interaction are by no means a cure-all for stress, yet they can and often do aid in refocusing the mind on tasks that can be managed and controlled rather than sources of stress from the players real life. Moreover, the three genres we will discuss here deal with time in much the same way in relation to their gameplay mechanics. All three allow the player to have interesting and meaningful interactions with the simulations or gameplay systems while at no point relying on physical reflexes, dexterity, or mental processing speed. Three genres which allow for this type of gameplay are city builders, 4X strategy, and puzzle games.

City Builders

City builders are perhaps one of the most logically named subgenres to be found within the greater grouping of simulation games. Simulations as a whole are games which seek to emulate real-world systems such as flying a plane, driving a car, or running a farm. In city builders, players are tasked with managing a city, theme park, hospital, prison, or any number of other complex and interconnected systems that require management. One of the major traits of this subgenre is the isometric overhead interface. Players are able to see from above the entire structures or series of structures they are creating, thereby removing unknown factors from the gameplay. In other words, almost no information in city builders is hidden from the player; rather, challenges to the player can generally be anticipated and dealt with. This stands in stark contrast to two of the other major game genres that utilize the isometric map view for primary gameplay – namely, the real-time strategy and role-playing game genres. In these often highly stressful genres, most of the map starts out hidden by the “fog of war” until the player sends units out to explore the area. Moreover, the unknown areas are full of enemies waiting to attack the player.

City builders, however, have no “enemies” to speak of aside from simulations of the challenges that would often face a city.

From a historical perspective, the city builder genre has seen a resurgence in the past decade after a number of years out of the limelight. The company Maxis pioneered this genres in the late 1980s with the hit game *Sim City* and its sequels. The franchise was later eclipsed by the popular spinoff *Sims* series which diverged from the city builder genre and produced *The Sims 4* in 2016. With Electronic Art’s purchase of Maxis in 1997, the supremacy of the studio in the city simulation genre begin to diminish. Today, competing companies like Frontier Developments and Paradox Interactive are leaders in this subgenre.

The success of Paradox’s *Cities: Skylines* reflects many fans desire for an ultimate sandbox where players can create to their hearts’ content with almost no limitations. Interestingly, while *Cities: Skylines* allows players to deal with the challenges of managing a budget while at the same time trying to increase their city size, many players found more enjoyment in taking advantage of the built-in option to allow infinite finances to create the city of their dreams. In this style of gameplay, the goal of the game is drastically shifted. No longer is the aim to acquire enough finances to grow the city, but rather it becomes whatever goal the player sets for themselves. With the addition of expansions ranging from mass transit to an arctic setting, the game continues to add toys to this virtual sandbox. While some gamers doubtless find the lack of challenging gameplay boring, others take advantage of the creative outlet to test new ideas and theories (Gandolfi 2016). The game presents them with complex yet manageable problems, such as resolving traffic backups on a roadway, with no imposed time limits and the ability to pause time at will. This then gives the player a calm environment to focus on a single problem and come to a well-reasoned solution. The fact that the game is constructed to allow the player to focus on a single task at a time is integral to the potentiality that this genre is helpful to those suffering from high levels of stress. The feeling of overload and being out of control that accompanies a panic attack is in many ways reduced by focusing on a single, manageable task with a rewarding, yet open ended conclusion.

Another trait of the game which could potentially aid in dealing with anxiety is the fact that the problems that the player is tasked with overcoming in *Cities: Skylines* can in nearly every case be anticipated, assuming the player is familiar with the game. There are no surprise ambushes or unexpected complications. For example, considering the issue of traffic management again, the player can expect that building high-density areas will cause traffic congestion and thus build roads accordingly prior to creating the high-density area. The idea being that planning ahead to preempt a problem rather than rely on reflexes to solve a challenge may be a much more satisfying gameplay mechanic for those who suffer from high levels of stress.

As noted before, many simulations that fall within this categorization do not actually involve building a city. Frontier Developments was formed by the team behind the original *Roller Coaster Tycoon* (RTC) series after the IP was acquired by another studio; ironically putting their new game – *Planet Coaster* – in direct competition with a reboot of the *Roller Coaster Tycoon* series, *Roller Coaster Tycoon World*. The competition however was short lived when the RCT reboot was met with dismal reviews and bug laden gameplay. These theme park management games task players with creating and managing an amusement park, giving them the tools to create roller coasters as well as many other theme park attractions. *Planet Coaster* met with significant success and reflected many of the same game modes as *Cities: Skylines.*

One aspect that stands out in *Planet Coaster* is the player's ability to exercise a high degree of creativity. While the game does have financial management modes much like others in the genre, *Planet Coaster* allows an infinite money mode much like *Cities: Skylines*. This moves some of the gameplay challenge away from problem-solving and into pure aesthetic creation. Complete creative freedom is certainty nothing new in gaming. The popular game *Minecraft*, for example, contained a creative mode wherein players could arrange the games materials to create everything from a medieval castle to the *Starship Enterprise*. What *Planet Coaster* does do, however, is reward players for that creativity through the overlay of simulation. For example, if a player were to spend a significant amount of time

creating a building or prop with many elaborate moving parts, the simulated visitors to the park will have a more positive experience, thereby increasing future park attendance. The same could be said of the perfect coaster track design or food stall creation.

The significance here is that positive feedback to the player comes not from reaction time or combat, but rather pure creativity. The pull of video games for many is positive feedback – either in the form of 'winning' or in the case of city builders creating thriving ecosystems. If positive mental feedback can be achieved without the adrenaline-inducting gameplay mechanics found in many other game genres, then perhaps the calming and thoughtful gameplay of *Planet Coaster* as well as other games of the genre could be of use to those suffering from anxiety disorder. City-builders are not the only genre which possess traits that lend themselves to thoughtful and often calming gameplay. Another grouping of games that is admittedly more stress inducting but also more focused on problem solving is the 4X Strategy Game genre.

4x Strategy Games

The term "4X" stands for the four primary tasks of the player in games within this genre – explore, expand, exploit, and exterminate. Given these pillars of gameplay, it is clear that this genre moves away from the peaceful creativity of the city builder. However, it is important to note that the presence of violence in a game does not necessarily constitute a more anxiety inducing experience. Indeed, depending on the game mechanics, plotting the demise of an enemy and successfully excusing the strategy can be much more like solving a puzzle than a heated battle.

From a technical standpoint, a majority of these games have similar interfaces to city builders. Players generally spend a bulk of their time in game looking at a large map of the play area. This type of play area could be anything from a map of the earth to a depiction of an entire galaxy. A major trait of this genre however is that the player is tasked with overseeing far larger and often more and complex interconnected systems than in city builders. Moreover, one of the primary differences is the presence of actual opponents in the form of AI or potentially human controlled opponents. Where the city builder rewards creative

thinking, the 4X genre is designed for masters of strategic thinking.

The genre itself has a long history in video games. Titles like Sid Meier's *Civilization* series, beginning in the late 1980s, have spawned five sequels and shows no real signs of weakening. The *Masters of Orion* series as well has had a number of iterations and brings a similar style of game design to a science fiction setting. Other more recent titles such as *Europa Universalas* and *Stellaras*, both from Paradox Interactive, have made slight changes to the genre but overall still contain the 4X core systems.

In regards to those systems, one of the main relevant aspects that city builders and 4X games share is the lack of real time gameplay. A majority of 4X games fall into the categorization of turn-based systems. This type of game plays out exactly as the name suggests – the player takes a turn moving all of their game pieces, making all relevant choices with no time constraints, and finally deciding when to end their turn. At this point, the AI in the game moves all of their pieces, makes their choices, and so on. Indeed, this type of digital game is perhaps more like a traditional board game than any other. More recent titles have diverged slightly from the turn based system. The space empire title *Stellaris* for example is not turn based, but plays out in real time and it is up to the player to control the speed at which time passes. Further, the player can in fact pause the game while still being allowed to issue orders to units and make other meaningful choices. However, this structure can also lead the player to a feeling of being overwhelmed later in the game with too many events and crisis happening at once.

Along these same lines, 4X games are not devoid of potentially anxiety inducting stimuli. The consistency with which the player must make complex choices can, over time, move from being strategic in a relaxing way to a level of complexity that is stress inducing. Moreover, these games are often equipped with intelligent AI which, along with the themes of eminent destruction, can make the games tense as well. This, coupled with the fact that players don't know what the AI is doing outside of their own territory in the game, can make for some interesting – and at times stressful – surprises. However, despite the stress that could potentially be induced from an

unexpected attack by the AI, these stressors are distinct from other game genres in that they do not depend on reaction time. The player may be surprised by an action of their AI opponent, but given that it is not their turn, there is no action to be taken. Moreover, when their turn starts, they have as much time to consider all their options as they would like. Thus, while this type of gameplay can indeed be stressful, it is decidedly different from the fight-or-flight response that can prompt a panicked reaction. This design choice of allowing the player indefinite time to process the challenges put forth by the game is a major aspect shared by the puzzle genre.

Puzzle Games

The use of puzzles in video games is hardly a new development. Indeed, many of the gaming industries earliest successes were rooted in this genre. Early titles like *Zork* and *Adventure* combine narrative and puzzle aspects, while later PC games of the 1990's such as *The Fool's Errand* and later *Myst* allowed progression through critical thinking skills in differing forms. While this genre is not as popular as it once was, games like *Portal*, *The Witness,* and *Obduction* continue to enjoy a following among gamers. This genre of games is in many ways difficult to categorize in that there is a great deal of variation.

The previously mentioned *Myst,* for example, integrates puzzles into the virtual environment itself, asking players to discover how levers and machinery can open new paths to further their exploration as well as advance the narrative. They key here however is that these puzzles are not timed and are revealed to the player in a predictable way. In effect, while some games in this genre do have narratives, the gameplay of puzzle games exists entirely in the present. For example, a game character may be waiting in a dungeon for a player to solve a puzzle door in order to break them out, but the player can take as much or as little time as they like and the game character will react in exactly the same way. For the player, time stops when the puzzle begins. Players are free to spend as much time as they like considering all possible options and utilizing their critical thinking skills. This lack of a time limit creates a play experience wherein reflexes and potentially stressful time constraints do not factor into the game. The gameplay itself is the methodical

discovery of rules which govern the puzzle process and ample time in which to mentally process the solution. Players rarely need to concern themselves with factors from outside the context of the puzzle itself.

Another aspect which may suggest the benefits of puzzle games to those suffering from high stress is the level to which they require intense mental absorption while avoiding dependence on reflexes that many other genres require. At face value, it may seem contradictory to suggest that mental absorption is both helpful and harmful to those suffering from high stress levels. However, as Kircanski noted, GAD involves "multiple sources of potential threat" (Kircanski et. al. 320). When a player is faced with a single threat or challenge, as in a puzzle game, it allows them to devote their entire focus on the problem at hand. This not only makes for a less stressful gaming experience, but more importantly could potentially aid players in redirecting the "pervasive, excessive, and uncontrollable worry" related to other real-world problems that GAD can induce (320).

This is not to say that puzzle games have no negative outcomes, but while there are consequences that players could perceive as threats, this genre of games rarely has what Lieberman et. al. (2017) would categorize as uncertainty, or U-threat. In the game *Portal,* for example, players can be killed by neurotoxin if they incorrectly navigate a spatial puzzle. However, the knowledge of the threat is given to players in advance, thereby avoiding the surprise aspect. This potential for negative outcomes keeps the game interesting and exciting, but avoids the stress response common in many other game genres. Further, this aspect of narrative based threat motivates the player through curiosity rather than competition or the flight or fight response.

Puzzle games, however, can induce stress if they fail to maintain a good balance of challenge and progression. This balance, often referred to as "flow" among game scholars, is of particular importance in regards to stress and the individual personality of the player. For example, if a player is unable to crack a particularly difficult word puzzle in *The Fool's Errand*, it is possible that this inability to finish the task will lead to frustration. This, in turn, could lead to a spike in tension and move the game from being a relaxing activity to a stressor. Certainly, no one

enjoys repeated failure. However, where some players may embrace the patience needed for a puzzle game, others may find it irritating.

Genres That Hurt

While these genres of City Builders, 4X Strategy, and Puzzle Games could potentially lead to a relaxing experience, they are far from the most popular genres in gaming. Indeed, in our discussion of stress inducing games to follow, it is important to keep in mind that high intensity genres represent the bulk of the gaming industry. Games like *Call of Duty*, *Fortnight*, *Resident Evil IV*, and others lead the way in global sales. Many games in the following genres fall into what Schmidt et. al (2013) categorized as action games. That is to say, games "involving hand-eye coordination movements through controllers or motion/gesture based systems" among other traits (1450). As noted in the literature review, action games also seemed to be linked to higher stress and worry levels in the 2013 study. This then leads to the questions of what exactly it is about these genres that potentially increases stress.

First Person Shooters

First person shooters could be described as the poster child of violence in gaming, being what most people think of when the topic of video games and violent behavior comes up. Games in this genre put the player into a 3D environment and arm them with a weapon of some kind. Then, as the genre name implies, the player shoots at enemies in order to complete missions or to win matches depending on the context.

The popularity of games in this genre has eclipsed most others, with hits like *Call of Duty*, *Bioshock*, and N64s *Goldeneye* being benchmarks in gaming history. As broadband speeds increased in the mid 2000s, online competitive shooters began to pull in millions of players every year. Today the first-person shooter genre continues to be one of the most popular and a major vehicle for the growing area of esports with titles like *Overwatch*.

While this genre is loved by millions of gamers, it also contains traits which may make it harmful to those diagnosed with high stress levels. One of the primary issues with games of

this genre is their reliance on rapid sensory processing by the player. Shooters, particularly when in online play, move at incredible speed and require lightning fast reaction times in order to progress. As previously noted by Goddard, those suffering from PD often struggle with sensory inhibition (Goddard 6). In other words, the overwhelming sights and sounds could potentially lead to the overstimulation of the players brain in a way similar to a panic attack. This is not to say that players actually believe they are in danger from the game, but rather when they are relying so heavily on their adrenaline for increased reaction times, that same adrenaline can be produced out of control in those suffering from PD.

This aspect of shooters also ties into the idea of time as an integral factor. In simulating the real-world physics of the battlefield, games require real-time reaction speeds on the part of the players. While strategy does factor in to some extent, it must be implemented with dexterity and skill, often in fractions of a second.

Platformers

Based on their often family friendly aesthetics, games in the platforming genre may seem unlikely to increase stress levels. Many of the most popular games of all time, including the *Mario* series, fall into this genre. Granted, it has changed to some extent since its height of popularity in the 1990s with the introduction to the 3D space along with other advancements, yet at the core it remains the same.

The challenge of the gameplay in this genre comes entirely from player movement. Running and jumping from platforms (thus the name *platformer*) is the core gameplay. These games also have enemies which the players must defeat, but often those are dealt with through player controlled movement as well. The key aspect of stress in this genre however relates to the reaction time and dexterity required of the player. Often, the act of jumping from platform to platform demands precise timing and intense concentration.

This level of gaming intensity is often exacerbated by the consequences of failure in these games. Where many recent first-person shooter games such as *Bioshock* and *Call of Duty* let the player retry a level or mission as many times as they would

like, platformers often give player three lives or less. Three chances to perfect their gameplay is all the player is afforded before having to restart the level, or sometimes even the game itself. Further, classic platformers often have a character die at their first injury or mistake rather than have a health bar of some kind. In the *Mario* games, for instance, touching an enemy a single time will kill Mario and take one of your three lives.

This ties in to stress on several levels. First, the consequences for lack of threat response is extremely high within the context of the game. Players are faced not with a minor setback, but rather with the prospect of losing multiple hours of gameplay. Secondly, the U-threat levels for platformers can be extremely high. Many games in this genre expect players to die on their first and second attempts due to unforeseen threats blindsiding them. The concept is that players will memorizing the layout of the level on these first attempts, then on later attempts, they will remember exactly when to dodge, jump, or run in a way that allows them to finish the level. However, for someone suffering from heightened stress levels, the ongoing and very real perception that at any moment they may encounter a startle response triggering event can lead to overall difficulty in dealing with games of this genre.

Survival Horror Games

Perhaps the least surprising of genes that prompt anxiety, games in the survival horror genre pull heavily from tropes found in other mediums. Where the characteristic of gameplay begins to branch off however is in the aspect of survival. Many games in this genre focus not only on avoiding direct threats such as monsters or other entities, but also on managing resources such as light, food, and water.

Popular recent titles in this genre include *Resident Evil VII*, *Amnesia*, *Outlast*, and *Alien: Isolation*. It is important to note that other recent horror games like *Until Dawn* certainly fall within the horror genre, and induce stress in much the same way as a horror film, but lack the survival aspects of true survival horror.

The theming of this genre however generally follows the same tropes as horror film. A player is left in a dangerous and usually abandon location, and must find a way to survive their situation when faced with a malevolent threat. The threats come

in many shapes and sizes, but for the most part can be categorized as some type of monster or evil intelligence. One of the key aspects of the genre is that players rarely, if ever, have a direct way to challenge or defeat the threat.

In relation to anxiety levels, games in survival horror are focused entirely on U-threats. Indeed, the gameplay itself revolves around being frightened by an unexpected threat, dealing with that threat (generally through running away), and then, before the player can collect themselves, being faced with another unexpected threat, and so on. The title of the genre itself – survival horror – can be seen as a description of the gameplay. Players are not asked to 'win' – the best they can hope for is to merely survive. Moreover, in contrast to most shooter games, the fact that players have no way to fight the threat presents a limited set of options when faced with a U-threat. When the player has no option to fight within the game, they can only flee. This then leads to incredibly tense sections of gameplay wherein the player is being hunted by the game antagonist, often being forced to hide and hope they are not seen. For those without an inclination towards high stress levels, this experience can provide a thrill, much like a roller coaster or other activities that induces an adrenal response. Herein lies the appeal of the genre. However, as noted, U-threats in those with PD trigger an adrenaline-fueled fight or flight response; a response that can quickly become overwhelming and move beyond direct connections to the game world.

Much like other situations wherein PD sufferers are unable to quell the adrenal feedback loop, survival horror games could potentially lead to a panic attack experience. While the horror genre itself, relies on jump scares the intentional induction of adrenal response, the survival horror genre adds another layer of stress on top of that. Not only are players keenly aware of reaction time, ready to rely on reflexes to save them from a direct threat, but they also must be acutely aware of how much time their resources will last. This can come in many forms, ranging from a flashlight battery in *Slender Man* to the amount of sanity the player loses when looking at something frightening in *Amnesia: The Dark Decent*. The key aspect in all of these genres however is time. Time is used to create tension, but for

those with high stress reactions, that same mechanism can lead to harmful responses outside of the gameplay experience itself.

Conclusion

The primary purpose of video games is to entertain the player. Granted, many argue that they are everything from an art form to an addictive drug, and the ways in which games are used is constantly evolving. Yet, at their core, they remain something that people do for enjoyment. If, as suggest here, certain genres of games could be harmful to those who are already suffering from high levels of stress, while others could be helpful, what implications does this have for future research?

The first major step in better understanding what impact game genres have on players would be to conduct a study in which the biological responses of players are tested when playing games. This type of study has been conducted before in average players and has indeed shown that games have a physiological impact on the human body (Bouchard et. al. 2014). However, a direct study of those suffering from stress disorders such as GAD or PD and their reactions to the genres discussed would be beneficial. A study of this sort would benefit from a qualitative pilot study followed by a quantitative study measuring responses to the games on a physiological level.

In looking at greater implications, if the genres that help can genuinely aid those suffering from PD or GAD, they may prove an effective tool for therapist to use in treatment of patients. This is not to say that video games could replace needed medications or therapy, but prescribing particular activities or exercises in the treatment of GAD and PD is hardly unprecedented. For example, recent studies have suggested the effectiveness of online based therapy for these disorders (Kumar, Bell, & Juusola, 2018). Games, however, allow for an extremely directed experience wherein the therapist would know exactly what the player would encounter while simultaneously engaging the patient in activities they may be doing already. This could potentially lead it to be utilized as a more effective treatment tool.

The aspect of time and stress in gaming also could be of use in treatment as a form of exposure therapy which is also successfully used in treatment of anxiety disorders (Anderson, Edwards, & Goodnight 2017). Tasking patients to play games

wherein they cope with time limitations as discussed in this study could gradually aid them in overcoming their fight or flight reactions to those types of situations in the real world. From an autonomic perspective, the body is reacting in a similar way to simulated stress as it is to real world stress. Ergo, slowly acclimating to the stresses of shooter or platformers may help those with high stress levels cope with similar feelings in the real world. This is not to say that most patients would be shooting weapons or jumping from platforms, but many real-world activities ranging from driving to social conversations depend on reacting to sensory input in within time constraint.

While games could potentially be used as a form of treatment, it is also important to consider that players should be aware of how games might affect them. The irony of having high levels of stress is that games are often thought of as a stress reliever and players may not realize that the game is significantly affecting their stress reaction in a negative way until after the effect has begun. They may be having fun, focused on the game, but then suddenly realize that their heart rate is increasing and are on the verge of a panic attack. This certainly does not apply to all who suffer from high levels of stress, but those who know they are predisposed towards these issues would do well to consider their current mood, life situation, and stress levels before deciding which title from their game collection to play.

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A Call for the Use of Geophysical Techniques to Monitor Landfill Leachate

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Abstract
The ecological implications of groundwater contamination are unbounded. Private citizens, environmental advocates, and industry alike all have stakes in maintaining a sound hydrologic system. In determining contamination of groundwater from landfills including those containing fly ash (a coal combustion by-product), electrical resistivity tomography (ERT) can be used as an economical way to analyze existing landfills to map potential karst features and identify probable groundwater seepage through and beneath the landfill.

Introduction

Since the emergence of the industrial age, humanity has been struggling with the issue of appropriate disposal of waste from manufacturing and mining processes. History is littered with examples of ecological disasters brought about by the careless handling of hazardous materials and to be fair, in the early days of this movement, many of the dangers associated with certain processes were simply unknown. Humankind can no longer assume such ignorance. Today, people throw away trash at an alarming rate and continue to use fossil fuels as a significant source of power resulting in the need for appropriate and safe disposal of the waste products associated with their use. While mankind may not entirely rid the world of fossil fuel use or solid waste, society can at least be more diligent in protecting the environment from the repercussions of their use and employ better methods of stewardship for the planet. Being aware of the need for proper landfill design and monitoring of leachate with more cost-effective measures is the first step.

Design and Construction of Fly Ash Landfills

Landfills are physical constructions designed and built for disposing of solid wastes in the earth's surface soil to avoid polluting the surrounding environment and the groundwater. Fly ash landfills are a particular type of landfill constructed and designed to accommodate the by-products of coal combustion residues, which contain toxic materials such as heavy metals, trace elements and soluble oxides of chemical elements that are of primary concern to environmental, governmental, and community bodies in the contamination of groundwater pollution and subsurface soil. Increasing production of fly ash from coal-based industrial processes like power plants is posing a challenging problem concerning its safe disposal and proper utilization ("Frequent Questions About", 2018). Fly ash is rich in leachable heavy metals such as arsenic and boron, which are considered highly hazardous materials according to the environmental regulations in most of the countries of the world (Deonarine, Kolker, & Doughten, 2015). Fluctuating water tables and rainwater interacts with fly ash in landfills, causing the leaching of toxic heavy metals and trace elements to the subsurface soil and groundwater, thus leading to pressing environmental issues. As such, it becomes mandatory to characterize fly ash, along with the identification of its leaching characteristics to adopt efficient disposal strategies (Singh & Kolay, 2002).

Fly ash landfills are constructed to eliminate the visual and non-visual environmental threats of trace elements, heavy metals and other element oxides that are generated from the combustion of the different types of coal. They are also intended to prevent these toxic and hazardous materials from migrating to the groundwater or being retained by subsurface soils and ultimately reaching the food chain, which then impacts human life and living organisms. Landfill designs are all meant to be secured, but for fly ash landfills, the secure construction requires a number of elements to be considered that include (i) a bottom-liner system (consisting of a compacted clay layer and/or geosynthetic layers) at the base and sides of the landfill to prevent the escape of the leachate material to subsurface soils and groundwater, (ii) leachate collection pipes to collect the leachate from the landfill base and to prevent the leachate

clogging, (iii) geotextile filter layers to filter or act as a pipe to drain the leachate to the removal area, (iv) a final cap of cover soil to divert the surface drainage away from the landfill, (v) landfill structural stability, and (vi) a hydrogeological setting and surface water drainage system to minimize the chances of washing the landfill materials into the natural drainage system, as seen in Figure 1 (“Duke Energy to Build”, 2015).

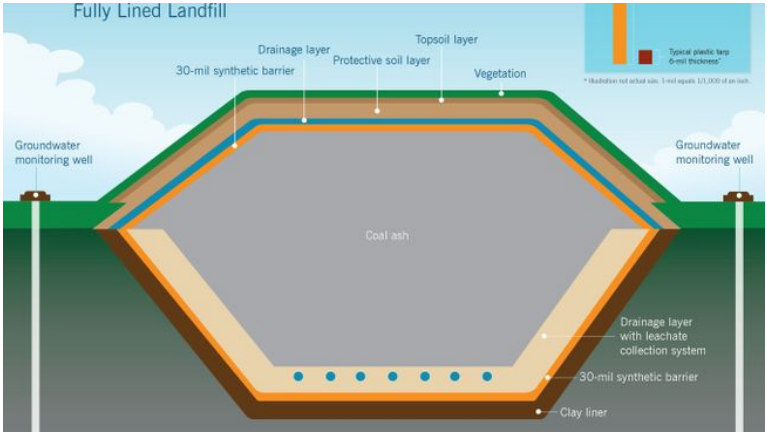

Figure 1. Elements of design of coal ash landfill (“Duke Energy to Build”, 2015).

The liner system in landfills is the principal element in the design, as it secures the leachate and prevents it from reaching the groundwater and subsurface soils. Many developments have occurred in designing, constructing and maintaining liners for a variety of purposes. The main types of liners include clay liners, geosynthetic liners (made from polymer materials), and geosynthetic clay liners (a mix of geosynthetic and clay components). Compacted clay material is used as a classical liner in landfills due to the relatively low hydraulic conductivity (10-7 cm/sec) that characterizes compacted clay (Das, 2014). In 1987, the Environmental Protection Agency (EPA) enforced a minimum of a double-liner system design for leachate collection and removal (EPA, 1985). Before the enforcement of double liner systems, single clay liners or single geomembrane liners were commonly used for landfills.

Permeability and hydraulic conductivity (K) are often interchangeably used terms for the same property. Permeability (K) is defined as a measure of the amount of fluid that will flow through a sample for a given time without causing displacement. Fluids flow through the void space, not the particulate matter, and that gives porosity a controlling influence on permeability. Successful compaction of the clay layers is essential so that the permeability is reduced for both the liners, the clay, and between the compaction levels; otherwise, leachate could escape through the compaction lifts interfaces.

The Leachate Removal System

The leachate in landfills is caused by the washing down of the landfill materials by rainwater, snowmelt and flood water into the subsurface, which represents a major source of pollution to groundwater and subsurface soils; therefore, proper containment is imperative while constructing and designing landfills equipped with double-liner systems to avoid the anticipated hazards of fly ash leachate in fly ash landfills.

The leachate removal systems employ pumping or gravity to remove leachate from the base of the landfill efficiently. Thus, the proper gradation of the landfill floor is a vital design element to drain leachate to the collection and removal point (Figures 2 & 3).

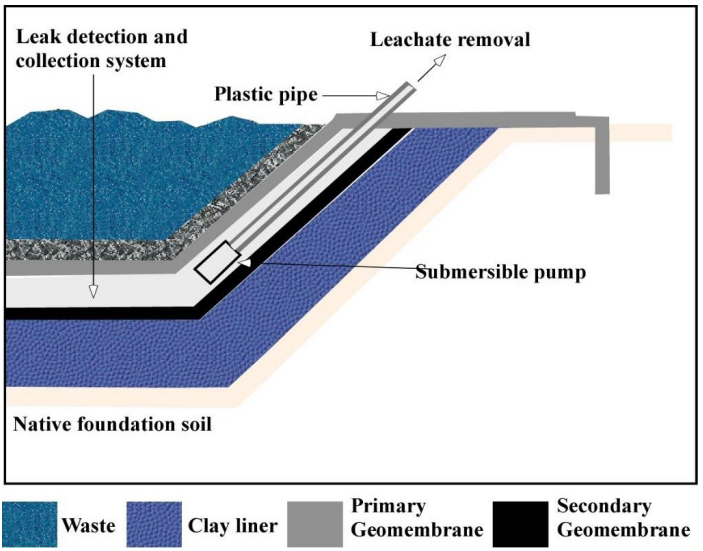

Figure 2. Secondary leak detection, collection, and removal (LDCR) system, utilizing pumping (Note: The plastic pipe penetrates the primary geomembrane) (Das, B. M., 2014).

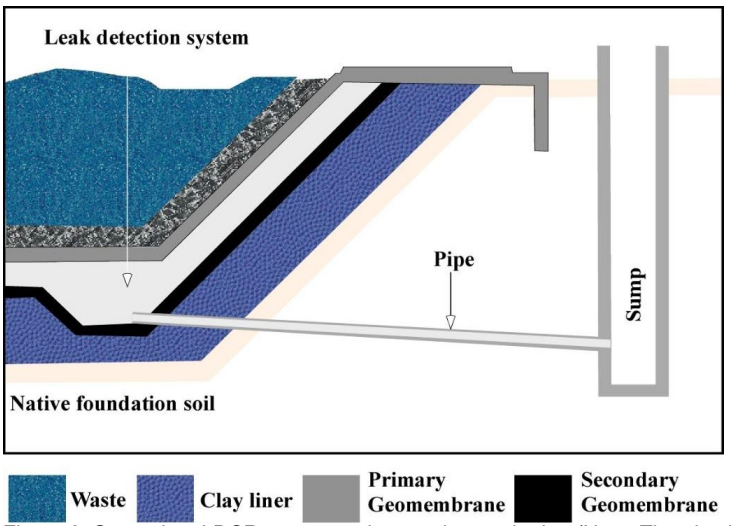

Figure 3. Secondary LDCR system, using gravity monitoring (Note: The plastic pipe penetrates the secondary geomembrane) (Das, B. M., 2014).

Closure of Landfills

At the end of the age of the landfill, a cap cover is placed for minimizing the production of leachate and minimizing the infiltration. As a result, this protects the groundwater from being contaminated. Clay-rich soils are used in constructing low-hydraulic-conductivity covers for landfills (Daniel, 1993). The cap cover is constructed of layers of compacted clay, topped by geomembrane and drainage layer and finally, covered by topsoil. The upper topsoil allows water to percolate to the protective impermeable layer or geomembrane. The function of this protective layer or geomembrane is to direct the water flow laterally away from the fly ash landfill. The diverted stormwater will be collected at the designated drainage system at the toe of the landfill (drainage ditch), and eventually, be directed to the natural drainage system and the low area of the landfill where the retention pond is located. Stormwater that collects via the drainage ditch will reach to the groundwater, bearing no contaminants, while on the other hand, the protective soil layer or geomembrane will prevent stormwater from making contact with the leachate of the landfill.

Fly Ash Landfill Stability

Landfill stability is controlled by many factors that individually or combined can lead to failures. These factors include the angle of slope, the nature and the properties of the base and liner's sides and the interface material, the height and weight of the material and the change of pore pressure. These factors affect the stability of landfills by changing the shear strength. The stability of the landfill liner system is affected by the interface shear strength between the geosynthetic materials; the interface shear strength between geosynthetic and soil materials; the internal shear strength of geosynthetic clay liners; the internal shear strength of solid waste and on the slope and height.

Stability analysis studies of fly ash landfills showed that the dominant landfill failures occur at the interfaces between the different liner planes of the base and along the side slopes in a composite liner system (Bergado, Gunturi, Sia, & Varun, 2006; Dixon & Jones, 2006). Failures of bottom liner landfills induce leachate leakage into subsurface soils and groundwater. Malfunctioning and clogging of leachate draining systems can

lead to spilling over the landfill sides, change of pore pressure and pressure on the bottom liner, or both, and consequently, increase the probability of failure. Leachate can leak in the bottom of the landfill when a failure of the bottom liner happens, or it can fill up the landfill and spill over its sides. If the landfill fills up with fluid, this fluid weight puts pressure on the bottom liner, which will lead to bottom liner failure. Other reported failures include sudden sinkhole collapses in karst terrains. The existence of either pre-existing air-filled voids or pre-existing or newly infilled clay-filled voids can seriously affect the landfill stability and causes failure.

The Impact of Fly Ash Landfills on Hydrologic Systems

Fly ash in most landfills comes eventually in contact with water and generates leachate, which passes contamination threats to hydrologic systems. Most of the efforts in landfill operations go to reduce the highly visible surface contamination problems, while concerns with the invisible impacts of fly ash on groundwater are growing (Ghuman, Sajwan, & Denham, 1999). The potential for groundwater contamination due to leachate from coal combustion by-product disposal sites was identified by the EPA (1988) as a primary concern, based on the elevated concentrations of soluble salts and potentially toxic trace elements. Evangelou, 1996, stated that coal ash is made of three types of solids (i) chemically water stable solids (SiO, FeO, AlO), (ii) relatively water soluble solids (e.g., metalSO4, metal-BO3), and (iii) water reactive metal-oxides (e.g., CaO, MgO, K2O, Na2O, etc.). On the other hand, Carlson and Adriano, 1993 showed that the effects of fly ash on groundwater quality depend on the physical and chemical characteristics of the ash, the hydrogeological conditions and the climate at the disposal site. Hence safe disposal of fly ash with respect to surface and groundwater protection depends on having the know-how to evaluate the potential of a given fly ash to release its toxic pollutants.

The leaching behavior of fly ash is a complex process and could be exemplified by the dissolution of lead. Understanding leaching behavior of fly ash and the leaching process is crucial in preventing the anticipated pollution and migration of the liquid that is collected at the bottom of the fly ash landfill (leachate) to

groundwater and subsurface soil and is vital in assessing and guiding the landfill disposal and detoxification of fly ash. (Gong & Kirk, 1994). The constituent of landfill leachate is water-laden soluble fly ash and heavy material, and the rain and surface water percolating through the ground. Different chemical and physicochemical reactions occur to transfer the fly ash constituents to the rain and surface water in a cation exchange process. The capacity and ability of soil to retain heavy metals are determined by the Cation Exchange Capacity (CEC) of the soil, which is defined as the number of milligrams of cations that are absorbed by 100 grams of the soil, and by the amount of minerals and organic colloidal matter present in the soil.

The diminishing concentration of the migrating leachate material through the subsurface is determined by the mechanical filtration, the amount of precipitation and surface water and the cation exchange capacity of the soil. Leachate collection systems are primarily included in the landfill design to prevent the migration of leachate generated inside a landfill from reaching the soil and groundwater beneath the landfill. This system works in conjunction with the aid of the liner system and methods to control and minimize leachate heads within the landfill and to prevent the damage of the liner system which is composed of compacted clays, geomembranes, and geosynthetic clay liners.

Case Examples of Landfill Failures

December 2008 brought an unwelcome arrival in Harriman, Tennessee. A coal ash dam at the Tennessee Valley Authority (TVA) Kingston Plant collapsed, liberating over 1 billion gallons of coal ash into two neighboring rivers, the Emory and Clinch Rivers (see Figure 4) (Smith, 2009). The visual provided in Figure 4 emphasizes the impact this disaster had on the ecology of the area.

Figure 4. Aerial images before and after the landfill failure in Tennessee, 2008 (Smith, 2009).

Other types of landfills can also experience difficulties resulting in the potential contamination of groundwater. A solid waste slide occurred in 2013 at the Big Run Municipal Solid Waste Landfill near Ashland, Kentucky (Figure 5). There, approximately 800,000 tons of waste slid over an area extending out some 121.92 meters off the liner. It was estimated that this 20-foot deep, 8-acre mound of waste was equivalent to nearly the annual amount of disposal (Gilbert, 2014). The forensic report of the landfill failure revealed that there had been a buildup and increase in pore pressure and development of wet spots along the soil cover that contributed to the failure, by inhibiting the draining of the abundant heavy rainwater. The absorption properties of the waste varied according to the waste type, hence, contributed to the failure by creating the soft wetted spots. The net result of the report is that water was trapped at the base, and the sides were the only avenue for escape; thus the slide took the garbage along with it.

Figure 5. Aerial view of Big Run, KY landfill slide failure (Gilbert, 2014).

A Cost Effective Solution

Failure of fly ash landfills caused by damage occurring in the liner or the leachate system could allow a wide variety of contaminants into the groundwater and subsurface soils, therefore, there exists a need to trap and collect water back into the leachate tanks. Fires might also erupt after failures of solid waste landfills, due to the gasses that escape the cover layer, allowing waste to make contact with air. This type of situation requires urgent treatment with sealant material to contain the gases. The cost of repairing and reconstructing these systems poses considerable financial and legal burden on companies and landfill owners, not to mention the environmental impact and potential human cost.

The imaging of landfills using ERT and MASW geophysical technologies could become an affordable way to reduce such costs. Electrical resistivity tomography (ERT) is a noninvasive geophysical technique that measures the electrical resistivities of subsurface materials, which depend on the lateral variations in subsurface resistivities. MASW is a non-invasive geophysical method designed to measure the spatial variations of the average shear-wave velocity of subsurface earth materials. The combined use of electrical resistivity tomography (ERT) and multi-channel analysis of surface waves (MASW) can provide a cost-effective means of non-intrusive geophysical profiling to detect anomalies in foundations up to 30 m (100 feet) beneath

the ground surface. These are useful techniques to map foundation conditions beneath existing landfill sites founded on karst terrain areas, and could be effective in multiple applications.

With the potential for disaster so great in association with failure of fly ash and solid waste landfills, it is imperative that new, more cost-effective methods of monitoring leachate be implemented. The cost in cleanup, human and animal life, and environmental stability are too high to risk inaction.

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Correlations between Gifted Middle School Students' Images of Mathematics and Their Images of the Elements of Mathematics: Foundations (EMF) Curriculum

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Abstract
This study explored the relationship between gifted middle school students' images of a self-paced, challenging, process-based curriculum, Elements of Mathematics: Foundations (EMF), and their images of mathematics. Survey research on gifted middle school mathematics students (N=39) determined that overall, most students believed mathematics is fun and thought-provoking; mathematics is connected to the real world; mathematics makes a unique contribution to human knowledge; and mathematics is a creative process that supports many different ways of looking at and solving problems. The average student enjoyed doing the EMF curriculum and strongly agreed that the curriculum had made them more ready for high school and college mathematics. Significant relationships between images of the EMF curriculum items and images of mathematics items were found. Those who enjoyed the EMF program and found it challenging and rewarding exhibited more positive images of mathematics.

Introduction

Almost thirty years ago, Clifford (1990) pointed out that it is a national crisis that most gifted students in public schools or traditional private schools do not have access to a curriculum that meets their learning needs. In 1990, Rothman surveyed some 25,000 gifted and non-gifted eighth graders and nearly 50% of them reported that they were bored in school half or most of the time. The same is still true today (Rogers, 2007). Gifted middle school students in most public schools and traditional private schools do not have access to curricula that allow them to go at their own pace, adequately challenge them, or present mathematics in a process-based way so that students can become familiar with behaviors of mathematicians (Gentry, Gable, & Springer, 2000; Rogers, 2007). Gifted students are subjected to textbooks and online materials that are not written at a level that is appropriate for them (Archambault, Westberg, Brown, Hallmark, Emmons, & Zhang, 1993; Rogers, 2007). Studies have also shown that many, if not most, teachers do not have the background or experience needed in order to engage and challenge gifted students (Westberg, Archambault, Dobyns, & Salvin, 1993).

Self-Paced Instruction

Gifted students often become bored and inattentive and may even become discipline problems when they are not given the opportunity to work at their own pace (Gentry, et al., 2000; Reis, Westberg, Kulikowich, Caillard, Hébert, Plucker, Purcell, Rogers, & Smist, 1993; Rogers, 2007). “Control appears to be a significant factor affecting student engagement in learning and quality of learning...and may serve to enhance relevance, achievement, and belonging for middle school youth” (Gentry, et al., 2000, p. 79). Many gifted students see classes that are led at one pace (even an accelerated pace) by a teacher as less productive and less enjoyable (Vialle, Ashton, & Carlton, 2001). Students in Vialle, et al.'s (2001) study remarked that they liked working independently “without the teacher interrupting” and that they don't “stare out the window so much” (p. 8). Rogers (2007) found that not only do gifted students prefer independent study, they actually find it stressful to be in situations where the learning isn't progressing according to their individual needs.

But simply allowing students to go faster is not a complete solution. Gifted students who are allowed to skip content or allowed to move up grade levels in school often report returning to the same level of boredom within 6-10 weeks of starting at the new level (Vialle et al., 2001). In addition to letting students work at their own pace, the curriculum must provide sufficient challenge and the environment must feel as if there are no ceilings on learning (Rogers, 2007).

Challenging Curricula

Although students should have access to curricula that are appropriate for their ability level, most textbooks, whether paper or digital, provide no challenge to gifted students (Gentry, et al., 2000). In fact, only about 25% of the pages in typical middle school mathematics textbooks even contain new content (Reis, et al., 1993) and half of the content in most mathematics curricula can be eliminated for gifted learners with no negative effects on student achievement (Rogers, 2007). Howley, Pendarvis, and Gholson (2005) state that schools "provide instruction that prepares few students for advanced" mathematics and therefore, few middle school students are "afforded experiences that position them to pursue...careers in mathematics or science" (p. 128). Rogers (2007) synthesizes an important lesson – gifted learners need daily challenges in the subject area to be mastered. Challenges need to be authentic, increase motivation, and develop higher-order thinking skills (Diezmann & Watters, 2005).

Challenge is essential for superior learning and building understanding (Gentry, et al., 2000; Rogers, 2007; Vygotsky, 1930-1934/1978). Vygotsky's (1930-1934/1978) theory about the "Zone of Proximal Development" found that keeping instruction just ahead of a student's developmental level to be key. Success has been tied to challenging tasks, high expectations, and conscious efforts to provide challenges often (Gentry, et al., 2000). Gifted students also report enjoyment of more challenging curricula (Howley, Pendarvis, & Gholson, 2005). For today's gifted students, this is not possible with most of the available curricula. Sadly, challenge is absent in many American classrooms (Archambault, et al., 1993; Clifford, 1990; Gentry, et al., 2000; Reis, et al., 1993).

Process-Based Curricula

Mathematical knowledge consists of more than just mastering rules and procedures (NCTM, 1989; NCTM, 2000; Thom, 1973). Mathematics is "the product of human inventfulness" (Romberg, 1992, p. 433). The idea that mathematics is a set of rules, handed down by geniuses, which everyone else is to memorize and use to get the "right" answer, must be changed. According to the NCTM (2000), understanding the processes of mathematics – problem-solving, representation, making connections, reasoning, and communication – are key to mathematical learning.

In his 1999 book, "Fostering Algebraic Thinking: A Guide for Teachers Grades 6-10," Mark Driscoll stated that it was critical for middle grades student to develop "habits of thinking" that are not unlike the habits of mind of practicing mathematicians. Students should be able to think about functions and the structure of systems. They should be able to build rules to represent functions and abstract from computation. Students should see proper modeling of algebraic thinking in their instruction. Students should develop a fluency in connecting and translating between multiple representations of the same concepts (Driscoll, 1999).

Baroody and Niskayuna (1993) posit that a problem-solving approach which focuses on the processes of mathematical inquiry (representation, problem solving, reasoning, and communication) is key in order to foster the development of mathematical thinking. Using inquiry and problem-solving strategies leads to deeper understandings in gifted students (Rogers, 2007). Fischbein (1990) states that mathematics educators need to "create an environment that would require a mathematical attitude, mathematical concepts, and mathematical solutions" (p. 7). Gifted students take more to higher-order thinking skills, learning to transfer the process skills they learn to new situations and other topics very easily (Rogers, 2007). Students who use process-based curricula come to have more positive and diverse beliefs about the nature of mathematics (Howley, et al., 2005). "There should be a qualitatively different presentation of content...for students who are extraordinary" (Rogers, 2007, p. 390).

Examining Gifted Students' Images of Mathematics

Attitudes, beliefs and views of the nature of mathematics, mathematical ability and mathematics education are all aspects of what Sam and Ernest (1998) call the individual's images of mathematics. When they enter middle school, both gifted and non-gifted students usually experience negative changes in motivation, self-concept of ability, attitude toward school, academic performance, and images of mathematics (Gentry, et al., 2000; Howley, et al., 2005).

Hrina-Treharn (2011) remarks that the research of gifted students' attitudes about mathematics is "limited and lacking" (p. 6). One of the few studies on the matter is that of Howley, Pendarvis, and Gholson (2005), which found that gifted students do not have very positive or nuanced views of mathematics. "Far from seeing mathematics as a way of expressing ideas or as a method for characterizing relationships and patterns, these gifted children instead saw mathematics principally as a set of procedures with numbers – as calculations and algorithms" (Howley, et al., 2005, p. 138).

What students believe about the nature and role of mathematics plays a major role in the learning of mathematics and research on students' images of mathematics is an important area to study (McLeod, 1992). Negative beliefs snuff out children's curiosity about mathematics and cause student motivations to learn mathematics to be based on grades and other extrinsic motivators (Howley, et al., 2005). According to Grossman, Smith, and Miller (1993) the most critical step in improving school mathematics learning is to change students' beliefs about mathematics. Successful performance on higher-order thinking tasks causes a more intense change in students' affective responses than successful performance on low-level computational skills (McLeod, 1992).

Learning should not just be an accumulation of facts. For meaningful learning to take place, students must have access to a challenging, process-based curriculum that is differentiated for their learning needs and exposes them to the true behaviors of a mathematician so that they can form deeper understandings of mathematics concepts and the nature of mathematics.

Connections between Gifted Students' Images of Mathematics and Curricula

At this point in time, no controlled studies have been completed that explore the effects of the use of self-paced, challenging, process-based curricula on gifted students' images of mathematics. If we expose gifted middle school students to self-paced, challenging, process-based curricula, we may change their images of mathematics (Rogers, 2007). This research seeks to establish whether there is a relationship between students' images of mathematics and their images of a self-paced, challenging, process-based curriculum.

Background

In the 1960s a talented team of curriculum developers began to develop the curriculum that is today known as *Elements of Mathematics: Foundations* (*EMF*) (IMACS, 2006). Kaufman, Fitzgerald, & Harpel (1981) wrote "we must not think that the role of mathematics in the school is essentially the same as the role of 'driver training'...If we are to serve the young well in our teaching of mathematics, we must ourselves have a thorough, humanistic, encompassing view of our discipline" (p. 1). Kaufman and his team started out with the process-based view that doing mathematics involved reasoning, making connections, problem-solving, representation, and communication – the behaviors of a mathematician – and developed a curriculum that was consistent with what the NCTM (2000) would later call the "Process Standards for Mathematics" and what the Common Core State Standards (CCSSI, 2010) would later term the "Standards for Mathematical Practice." Through the Ford Foundation, U.S. Office of Education, and Central Midwestern Regional Educational Laboratory (CEMREL) funding, the challenging curriculum was developed and the first students started to use it in 1966 (IMACS, 2006). Still today, there are classes using the paper textbook version of the curriculum with a face-to-face delivery model.

Starting in 2012, the self-paced, online version of the curriculum became available and is now in use by several school districts and by individual students from around the globe (IMACS, 2018). The mathematics content is presented in an integrated way and it addresses all of the Florida state and

national standards for Prealgebra, Algebra 1, Algebra 2, Geometry, Trigonometry, and Precalculus, and also includes many topics that are not part of the traditional K-12 mathematics curriculum. There are 18 content courses as well as some supplemental courses that prepare students for standardized testing.

Beginning in the summer of 2015, a large suburban district in Southeast Florida adopted the *EMF* curriculum for its most talented middle school mathematics students. Students who had been previously identified as gifted by the district that scored in the top 10% on state and district standardized testing at the end of their fifth-grade year were invited to begin the *EMF* program during the summer of 2015 as rising sixth graders. The students who successfully completed the first course of the *EMF* materials in the summer of 2015 continued on as the first cohort for this district to use the self-paced online *EMF* materials.

Research Questions & Methodology

The following research questions guided the research:

1. What images do gifted middle school students have of mathematics?
2. What images do gifted middle school students have of a self-paced, challenging, process-based curriculum?
3. What is the relationship between gifted middle school students' images of a self-paced, challenging, process-based curriculum and their images of mathematics?

To explore the research questions, a non-experimental, survey research design was employed. The survey instrument was developed by the researchers to collect the data. The chosen survey method was a questionnaire e-mailed to the study participants in the Spring of 2016. The respondents are 39 sixth grade students from a large suburban district in Southeast Florida that have been identified as belonging to the top 10% of gifted students in the district through standardized testing and who had participated as the first cohort in the district, completing the first block of *EMF* courses and requirements during the 2015-2016 school year.

Demographic items were chosen to reveal other pertinent connections. Respondents were asked to self-report their age,

gender, grades in mathematics and other subjects the previous school year (before joining the first cohort of *EMF* during the 2015-2016 school year), time spent on watching TV, electronic devices, reading, and homework each day, and favorite subjects in school.

Items from many (Aiken, 1963; Andrews & Hatch, 1999; Benbow, 1996; Brendefur, 1999; Carson, 1997; Coffey, 2000; Mitchell, 1998; Mura, 1995; Ruthven & Coe, 1994; Schoenfeld, 1989) studies about the various dimensions of images of mathematics were combined and modified to form the images of mathematics survey items. Additionally, these images of mathematics survey items were used previously on studies about teachers' images of mathematics (Goodwin, 2010; Goodwin, Bowman, Wease, Keys, Fullwood, & Mowery, 2014).

Items were included to determine if respondents were thinking of returning to the *EMF* program the following year and felt as if *EMF* had improved their confidence in their mathematical abilities, students' perceptions of the pacing and workload of the *EMF* program, the level of challenge in the *EMF* program, whether the *EMF* program had better prepared them for future mathematics classes in high school and college than a traditional class, and whether *EMF* had improved their ability to analyze complex problems, the ability to focus for extended periods of time, self-study skills, and confidence with respect to challenging programs.

Results

Demographics

There were 39 student survey respondents. About 73% of the student respondents were boys. Roughly 89% of the student respondents are 12 years old, with the remaining students being younger than 12.

The average student respondent reported that they were “confident” in their mathematical abilities. The average student reported that their grades in mathematics in the 2014-2015 school year were “Straight As” and their grades in other subjects last year were also “Straight As.”

The respondents reported that they spent 31-60 minutes on mathematics homework and watching TV each night, 61-90 minutes on reading and homework in all subjects daily, and 91-

120 minutes on electronic devices each day. The reported amounts of time spent reading, doing mathematics homework, and doing all homework are all significantly positively correlated. The average student agreed with the statement "I have enough time to complete all my homework each night." The level of agreement with the statement "I have enough time to complete all my homework each night" is significantly negatively correlated with amount of time spent reading and doing homework for all subjects.

About 84% of students declared mathematics as one of their favorite subjects. Science and music were also chosen as favorite subjects by about 63% and 55% of the students, respectively. Physical education and world languages were the least likely to be chosen as a favorite subject, with only about 21% of students choosing them.

Images of Mathematics

An overall image of mathematics item asks the respondent "Ideally, doing mathematics is like: cooking a meal, conducting an experiment, playing a game, doing a puzzle, doing a dance, or climbing a mountain." The most popular response by far was "doing a puzzle" with almost three-quarters of the responses. "Doing a dance" was not chosen by any respondents.

The students showed very positive images of mathematics. Students most strongly agreed with the statements "Mathematics supports many different ways of looking at and solving the same problems," "Math is thought provoking," "Mathematics makes a unique contribution to human knowledge," and "Math is intricately connected to the real world." Students also agreed with the statements "Mathematics is fun," "In mathematics, you can be creative," and "The process of trying to prove a mathematical relationship can change your mind about it."

Age of the student respondent was statistically significantly related to level of agreement with the statements "Math is intricately connected to the real world" ($t(35) = 3.81$, $p = .00$), "Mathematics supports many different ways of looking at and solving the same problems" ($t(35) = 2.94$, $p = .01$), and "In mathematics you can be creative" ($t(35) = 5.85$, $p = .00$). Those who were younger than 12 years old agreed more strongly with these statements.

Whether mathematics is listed as a favorite subject or not was significantly related to agreement levels with the statements "Mathematics is fun" ($t(37) = -5.15$, $p = .00$) and "In mathematics, you can be creative" ($t(37) = -3.73$, $p = .00$). Those who listed mathematics as a favorite subject reported that they "strongly agreed" with the statement "Mathematics is fun" on average, while those who didn't "slightly disagreed" on average. Those who listed mathematics as a favorite subject reported that they "slightly agree" with the statement "In mathematics, you can be creative" on average, while those who didn't "slightly disagreed" on average. Reported level of confidence in mathematical abilities is significantly positively correlated with level of agreement with the statement "Mathematics is fun" ($r(38) = .45$, $p = .01$).

Responses on the images of mathematics item "Ideally, mathematics is like" were statistically significantly related to whether a respondent reported mathematics as a favorite subject (χ^2 (1, N = 39) = 9.38, p = .00). About 84% of those respondents who said one of their favorite subjects was mathematics selected "doing a puzzle" on the images of mathematics item "Ideally, mathematics is like" while only roughly 29% of those respondents who did not say that mathematics was one of their favorite subjects picked "doing a puzzle."

The amount of time a respondent spends watching TV every night is significantly negatively correlated with their agreement with the statement "Math is thought provoking" ($r(39) = -.34$, $p = .03$). No other statistically significant relationships were found between demographics and images of mathematics variables.

Images of EMF

About 82% reported that their confidence in their mathematics abilities had improved since beginning *EMF*. The average student strongly agreed that "*EMF* is challenging," "*EMF* has made me more ready for high school than I would have been without *EMF*," and "*EMF* has made me more ready for college than I would have been without *EMF*."

Reported levels of enjoyment of *EMF* and other mathematics programs were significantly different ($t(38) = 2.66$, $p = .01$). The average student reported enjoying *EMF* at higher levels than they reported enjoying other mathematics programs.

The average student reported that the pacing and workload of the *EMF* program were "just right." Responses on the pacing and workload items were significantly positively correlated. All but two students reported that they want to return to *EMF* next year. The two that said they were considering not returning said they had hectic schedules and were not sure if they could fully commit to *EMF* due to time constraints.

The students agreed that *EMF* has improved their ability to analyze complex problems, their self-study skills, their ability to focus for extended periods of time, and their confidence with respect to challenging programs.

Relationships between Images of Mathematics and Images of EMF

Enjoyment of EMF.

Level of agreement with the statement "Mathematics is fun" is significantly positively correlated with level of agreement with the statements "I enjoy doing the *EMF* program" *(r*(39) = .65, *p* = .00) and "I enjoy doing other mathematics programs..." *(r*(39) = .37, *p* = .02).

Level of agreement with the statement "I enjoy doing the *EMF* program" is also significantly positively correlated with level of agreement with the statements "Mathematics makes a unique contribution to human knowledge" *(r*(39) = .47, *p* = .00), "Mathematics supports many different ways of looking at and solving the same problems" *(r*(39) = .44, *p* = .01), and "In mathematics, you can be creative" *(r*(39) = .39, *p* = .02). No other statistically significant relationships were found between the enjoyment of *EMF* and images of mathematics variables.

Confidence Levels after EMF.

Responses on the item that asked if *EMF* has improved confidence in mathematical abilities were significantly related to agreement levels with the statements "Mathematics is fun" (*t*(36) = -4.56, *p* = .00) and "In mathematics, you can be creative" (*t*(36) = -3.73, *p* = .00). Those who said that *EMF* improved their confidence in their mathematical abilities reported that they "strongly agreed" with the statement "Mathematics is fun" on average, while those who didn't "slightly disagreed" on average. Those who said that *EMF* improved their confidence in their

mathematical abilities reported that they “slightly agree” with the statement “In mathematics, you can be creative” on average, while those who didn't “slightly disagreed” on average. Responses on the images of mathematics item “Ideally, mathematics is like” were statistically significantly related to respondent’s views about whether *EMF* improved their confidence in their mathematical abilities (χ^2 (1, N = 38) = 5.32, p = .02). Almost 90% of those respondents who said “doing a puzzle” found *EMF* improved their confidence in their mathematical abilities while only about 56% of those who said something other than “doing a puzzle” reported that *EMF* improved their confidence in their mathematical abilities. No other statistically significant relationships were found between confidence levels after *EMF* and images of mathematics variables.

Ability Levels after EMF.

Perceived level of improvement in self-study skills due to *EMF* is significantly positively correlated with level of agreement with the statement “In mathematics, you can be creative” *(r*(37) = .36, *p* = .03). Perceived level of improvement in the ability to focus for extended periods of time due to *EMF* is significantly positively correlated with level of agreement with the statements “Mathematics is fun” *(r*(37) = .45, *p* = .01) and “In mathematics, you can be creative” *(r*(37) = .37, *p* = .03). Perceived level of improvement in confidence with respect to challenging programs due to *EMF* is significantly positively correlated with level of agreement with the statements “Mathematics is fun” *(r*(37) = .49, *p* = .00) and “In mathematics, you can be creative” *(r*(37) = .45, *p* = .01). No other statistically significant relationships were found between ability levels after *EMF* and images of mathematics variables.

Level of Challenge in EMF.

Responses on the images of mathematics item “Ideally, mathematics is like” were statistically significantly related to the reported level of challenge by *EMF* (*t*(37) = 2.04, *p* = .05). Those who said something other than “doing a puzzle” found *EMF* more challenging than those who said “doing a puzzle” The average “doing a puzzle” respondent was in the middle between “slightly

agreeing" and "strongly agreeing" with the statement "*EMF* is challenging," while the average non-"doing a puzzle" respondent strongly agree with the statement.
Responses on the images of mathematics item "Ideally, mathematics is like" were statistically significantly related to the reported change in confidence with respect to challenging programs ($t(35) = -2.21$, $p = .03$). Those who said "doing a puzzle" found *EMF* helped them gain more confidence with respect to challenging programs than those who said something other than "doing a puzzle." The average "doing a puzzle" respondent said their confidence with respect to challenging programs "is much stronger now," while the average non-"doing a puzzle" respondent said their confidence with respect to challenging programs "is slightly stronger now." No other statistically significant relationships were found between the level of challenge in *EMF* and images of mathematics variables.

Readiness Due To EMF.

Level of agreement with the statement "*EMF* has made me more ready for high school than I would have been without *EMF*" is significantly positively correlated with level of agreement with the statements "Mathematics is fun" ($r(39) = .48$, $p = .00$), "Math is thought provoking" ($r(39) = .47$, $p = .00$), "Mathematics makes a unique contribution to human knowledge" ($r(39) = .50$, $p = .00$), and "Mathematics supports many different ways of looking at and solving the same problems" ($r(39) = .50$, $p = .00$).
Level of agreement with the statement "*EMF* has made me more ready for college than I would have been without *EMF*" is significantly positively correlated with level of agreement with the statements "Mathematics is fun" ($r(38) = .44$, $p = .01$), "Mathematics makes a unique contribution to human knowledge" ($r(38) = .52$, $p = .00$), and "Mathematics supports many different ways of looking at and solving the same problems" ($r(38) = .50$, $p = .00$). No other statistically significant relationships were found between readiness due to *EMF* and images of mathematics variables.

Discussion of Finding

Overall, the views expressed by the student respondents about mathematics seem to be mostly positive. The average

respondent strongly agreed that mathematics is thought-provoking, connected to the real world, makes a unique contribution to human knowledge, and supports many different ways of looking at and solving problems. The student respondents found *EMF* to be enjoyable and challenging. Most of the respondents reported that *EMF* better prepared them for high school and college mathematics courses than the traditional curriculum. Many of the students reported that *EMF* had improved their ability to analyze complex problems, self-study skills, ability to focus for extended periods of time, and confidence with respect to challenging programs. Overall, the respondents who found *EMF* most enjoyable and rewarding agreed more strongly with many positive images of mathematics. As level of enjoyment of *EMF* increased, so did level of agreement that mathematics is fun, makes a unique contribution to human knowledge, supports many different ways of looking at and solving the same problems, and allows for creativity. Those that reported that *EMF* had improved their confidence in their mathematical abilities agreed more strongly that mathematics is fun and allows for creativity. Those that reported that *EMF* had improved their self-study skills agreed more strongly that mathematics allows for creativity. Those that reported that *EMF* had improved their ability to focus for extended periods of time or their confidence with respect to challenging programs agreed more strongly that mathematics is fun and allows for creativity. Those that thought *EMF* had made them more ready for high school or college mathematics classes than a traditional curriculum agreed more strongly that mathematics is fun, makes a unique contribution to human knowledge, and supports many different ways of looking at and solving the same problems.

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Teaching Girls to Program: 26 Years of Stagnated Thinking

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Abstract
This essay examines the state of Computer Science education and makes a comparison to the state of Computer Science education in the mid-1980s and early 1990s. There is a special personal emphasis on how girls see Computer Science education.

Introduction

Over two decades ago, I spent the summer after high school writing about my experience as a female trying to learn computer programming during middle and high school. At eighteen years old and about to leave for college, it never occurred to me that what I wrote would matter much. What I wrote was turned into an article called "High School Computing: The Inside Story" that was eventually included in a 1992 issue of *The Computing Teacher*, and while it was pretty cool to hold a hard copy in my hands (a published author at nineteen!), I was still too young to imagine how it could endure. Twenty-six years later, I am a mother of a twelve-year-old girl who also enjoys programming. With the wisdom and perspective that life brings, I find myself both amazed and dismayed.

I am amazed because, when Googling my old article to refresh my memory, I found that it actually had legs -- from *The New York Times* in 1992 (Lewis) to the International Conference on Computational Science in 2005 (Scott, Clark, & Bagheri) to two recent blog posts (O'Day, 2017; Winney, 2018). I am dismayed because, as Winney (2018) said of my article, "... it's still applicable today."

ME AT 18: "The root of this problem lies in what is currently thought to be important for students to learn in programming courses, namely, syntax" (Chen, 1992, p. 51).

Fast forward in time. My daughter recently attended a well-known, "creative tech" summer camp for girls. She has been programming for a number of years through online resources such as Scratch and eIMACS (IMACS, 2018), so neither of us expected her to learn a lot of new programming skills. We chose this camp mainly for the experience of living on her own on a college campus, and that part was executed brilliantly. Still, the camp description indicated that it would accommodate experience levels from beginner to advanced, and so we hoped that the programming exercises would be adjusted to her level.

ME AT 18: "The thing I liked most about taking this course in Scheme was that I knew that I was learning something" (Chen, 1992, p. 52).

Unfortunately, my daughter learned nothing new in the realm of programming at camp. The core project used a LilyPad Arduino kit and involved incorporating programmable lights (LEDs) into a hand-sewn prototype designed by the student to promote a social cause that matters to her (e.g. preventing animal cruelty). According to my daughter's description, the "creative" activities -- cutting, sewing, gluing and otherwise crafting material -- took up about 50% of the curricular time. Learning about circuits accounted for about 20%. The remaining 30% was split evenly between research and presentation skills and actually programming the LEDs.

My daughter improved her sewing skills, which, while handy, was not what she was seeking in 2018. She learned to build a circuit and a little about how circuits work. She got more experience with research and presentation, which is always practical. However, the extent of her coding was to declare and assign variables and to create a program that loops. The "advanced" instruction consisted of variations on programming concepts that she already understood but did not include teaching her any new concepts. I suspect that limited availability of advanced instruction was connected to the use of the C/C++-

based Arduino programming language. Syntax strikes again, only this time my daughter was the victim!

ME AT 18: "Concepts like these are universal in computer science. After you understand them, then you can learn the rules of any language in order to encode them" (Chen, 1992, p. 52).

Thankfully, my daughter is learning to excel at computer science by studying the updated, online successor to my old Scheme textbook through eIMACS (IMACS, 2018). Instead of pencil and paper, she has a laptop and WiFi in her study hall. But the time-tested approach of using Scheme to first learning computational thinking and the fundamental principles of computer science is still there and working as well as ever to nurture her love of the subject. She is also able to transfer her deep understanding of concepts into building creative and complex projects in Scratch and is looking forward leading her Girl Scout troop in earning the “Think Like a Programmer” badge. Her confidence in and enjoyment of programming stems from her strong foundation, which allows her to express her creativity and be a leader in more meaningful ways than tacking crafting onto coding.

I support the approach of showing girls how programming skills combined with creativity and communication can effect change. But we are never going to motivate more girls to learn computer programming with "girl-friendly" experiences so heavy on the creative that they don't get to feel what it's like have the true power of programming at their command. You don't have to sacrifice power for fun or vice versa. Choose a less-syntax-heavy programming language such as Scheme and the girls (and boys) we need to inspire to be leaders of change and innovation get to have both.

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Author Guidelines

The editor of *Journal for the Liberal Arts and Sciences* welcomes manuscripts related to a broad spectrum of academic disciplines and interests. Submissions should range from between 2000 and 5000 words in length, written in Times New Roman font (12 point) and must be accompanied by an abstract of up to 100 words. Manuscripts submitted for a special issue should include a reference to the theme of the issue. Authors can choose to submit their manuscripts as an email attachment to jlas@oak.edu or to the mailing address below. Email submissions are to include a message indicating that the manuscript is not under consideration with any other publisher but *JLAS*. Submissions by mail are to include a cover letter indicating that the manuscript is not under consideration with any other publisher as well as an electronic copy of the manuscript on either CD-ROM or diskette. All manuscripts must be submitted in MS Word format.

Receipt of manuscripts will be acknowledged within two weeks of submission. The review process generally takes between two to six months and authors will be notified of the editorial decision within six months of receipt. Total time between submission and publication can range from six months to one year. There is neither a remuneration offered nor a charge for publication in *JLAS*. Authors will be provided with three complementary copies of the issue in which their articles appear.

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Originals of tables and figures, such as graphs and charts should be supplied. Graphics should be prepared and captioned as exactly as they are intended to appear in the journal. All explanatory notes should be avoided whenever possible and the intonation incorporated in the text. Essential notes should be gathered in a section following the text and listed under the heading “End Notes.” End notes should be identified with consecutive numbers assigned in keeping with the superscript numeral used at the point requiring the note.

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