

ABSTRACT

Title of Document: EFFECTS OF 17- α -ETHYNYLESTRADIOL ON HYBRID STRIPED BASS SPERM.

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One of the most potent EDCs in the environment is 17 α -ethynylestradiol (EE₂), the hormone in most birth control pills. EE₂ is released into the ecosystem through human wastewater, affecting the environment and its inhabitants. Fish both live and reproduce in these affected ecosystems, which may make them particularly susceptible to the effects of EE₂. This study investigates the impacts on reproductive efficacy of acute, direct exposure of male hybrid striped bass sperm cells to EE₂. In the study, reproductive efficacy is measured by two endpoints: genetic integrity of sperm DNA and sperm cell viability. Genetic integrity and cell viability were assessed by the comet assay and SYBR-14/Propidium Iodide stains, respectively. The results concerning genetic integrity were not statistically significant, but the results of the sperm viability assay suggest that acute direct exposure to EE₂ does not cause significant death within a population of sperm.

Keywords: 17 α -ethynylestradiol, endocrine disrupting compounds, sperm

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By

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Chapter 1: Introduction

In 2002, a large-scale fish kill occurred in the South Branch of the Potomac River, just outside of Washington, DC. This event was followed by two others, in 2004 and 2005, in the Shenandoah River watershed. In all three events, over 80% of the adult smallmouth bass and redbreast sunfish populations were killed (Virginia Dept of Environmental Quality, 2010). Scientists suggest undue stress caused by man-made environmental disturbances produced such large-scale fish kills (Ripley et al., 2008). Such dramatic population losses garnered the immediate attention of scientists and wildlife experts. The already alarming events of 2002 took on a new sense of urgency when scientists inspected the fish more closely. Upon examining the gonads of the deceased fish, researchers discovered that many male smallmouth and largemouth bass showed intersex characteristics (Blazer et al., 2007). Intersex is a condition where an organism of one sex possesses sexual tissue of the opposite sex. Intersex can also manifest itself in the form of incomplete or mixed secondary sexual characteristics, or behavior changes that restrict or block courtship and mating. In the case of Potomac River bass, male fish were found to have immature oocytes present in their testes. This discovery helped to catalyze further scientific inquiry into the health of fish and other organisms in the Potomac River.

Through further study, it has been found that intersex fish are found throughout the United States. According to the United States Geological Survey (USGS), intersex fish were found in close to one third of all sites examined from the Apalachicola, Colorado, Columbia, Mobile, Mississippi, Pee Dee, Rio Grande, Savannah, and Yukon

River systems (Braun, 2009). In some cases, very high percentages of specific site's fish were intersex. In the Mississippi River at Lake City, Minnesota, for instance, 73% of smallmouth bass were intersex. In the Yampa River at Lay, Colorado, 70% of smallmouth bass was intersex (Ibid). These numbers are significantly higher than the percentage of intersex that is predicted to be naturally occurring: 4% (Ibid). Although these characteristics were found to be most prevalent among bass populations at these sites, they were also found in catfish (Ibid). The appearance of intersex fish has been documented in other wild fish populations around the world, including spot-tail shiners in the St. Lawrence River, roach in the U.K and Denmark, sharp-tooth catfish in South Africa, three-spine stickleback in Germany, and barbel in Italy. Intersex fish have also been found among marine and estuarine fish populations in Japan, the UK and the Mediterranean (USGS, 2008).

The presence of both male and female characteristics is not uncommon in all fish populations; there are certain fish that are natural hermaphrodites, possessing both male and female reproductive organs either simultaneously or at different points in their life cycle. Clownfish, for instance, are protandrous sequential hermaphrodites, changing sex from male to female throughout the course of their lives (Iwata et al., 2008). However, intersex fish are not hermaphrodites. The key difference is that hermaphroditism is an evolutionary adaptation meant to benefit the species as a whole, while intersex is a response to environmental stimuli that is generally considered to have adverse effects on populations in which it occurs. Unlike the sex organs of clownfish or other similar species, the intersex tissue in Potomac bass is non-functional and does not provide a biological advantage. Furthermore, the occurrence of intersex in Potomac smallmouth

bass is an anomaly. Smallmouth bass, like the vast majority of fish species, are not naturally known to display hermaphroditic characteristics.

Scientists have established that intersex is a unique biological condition distinct from naturally occurring hermaphroditism. However, this discovery only raises more difficult questions about the causes and effects of the condition. While the causes of intersex characteristics developing within an organism vary, the biological mechanisms through which intersex characteristics form are similar across species due to the highly conserved nature of the endocrine system. Despite its complexity, the processes and functions of the endocrine system are remarkably similar in all vertebrates (Ankley & Johnson, 2004). The endocrine system of fish is made up of many of the same glands found in humans, including the sex glands (ovaries and testes) that help control sexual activity and reproduction. Therefore, results gleaned from model species can be applied to other species of interest, including human beings. Compounds that alter the normal equilibrium of the endocrine system are known as endocrine-disrupting compounds (EDCs). EDCs can be synthetic or naturally occurring, but although certain endocrine-disrupting compounds are found naturally, the levels of these compounds in the environment is substantially increased due to human activity.

Anthropogenic sources of EDCs

Many substances can mimic estrogen's feminizing effects by binding to estrogen receptors in a variety of tissues. Human activity greatly increases the presence of these estrogenic mimics. Bio-waste from farm animals like chickens and cows seep into the

soil and the watersheds surrounding farms (Colluci, 2001). Estrogenic EDCs naturally occur in non-animal agricultural products like soy (Delclos et al., 2009).

Human birth control pills also contain concentrated doses of estrogenic EDCs (Daughton, 1999). Ethynylestradiol (EE₂) is a synthetic estrogen found in many birth control treatments. It is a comparatively strong estrogen and is almost twice as strong as biological estrogen (Gutendorf & Westendorf, 2000). While some of the compound is absorbed by the body, any excess dosage is then released into the environment through sewage. Many water filtration plants cannot remove molecules as small as estrogenic EDCs. This means that fish exposed to sewage runoff are exposed to estrogenic EDCs even if the water has been filtered. EE₂, the synthetic estrogen found in birth control, is estimated to have the single largest environmental impact of any individual EDC and is therefore of high concern (Johnson & Sumpter, 2001).

Humans have created a number of other synthetic estrogenic compounds contributing to the potential impact of EDCs on the environment. Many common industrial chemicals, pesticides, and plastics are estrogenic (Daughton & Ternes, 1999). In addition to compounds that are inherently estrogenic, some non-estrogenic compounds can degrade into estrogenic compounds, for example alkylphenols (Sonnenschein et al., 1998). The full range of EDC-breakdown products is not known, but the list continues to grow as more is learned about EDCs in the environment.

A high percentage of the estrogens and estrogen mimics come from nonpoint source runoff and sewage effluent (Kuster et al., 2004). Much water is needed to maintain agriculture and farming, and runoff from these operations often travels straight into lakes

and rivers. A high load of estrogenic compounds can come from these areas, including pesticides and fertilizers used on crops and hormones from livestock operations (Kuster et al., 2004). EDCs are also found in wastewater effluent (Snyder et al., 2001), coming from the array of chemicals used and expelled by humans, natural human waste, and the result of birth control pills. As previously stated, EDCs are often not removed during standard sewage treatment (Snyder et al., 2001). Increased exposure to estrogenic EDCs has been linked to negative externalities for fish, humans and other species.

Impetus for Experimentation

A great deal of information has been gathered on endocrine disruption since the issue first came to the attention of scientists in 2002. Although dramatic phenomena such as the fish kills of 2002 and the high prevalence of intersex characteristics in some fish populations are currently reserved for aquatic organisms that are constantly exposed to waterborne EDCs, scientists have quickly come to appreciate the potential ramifications of EDCs for terrestrial populations such as humans. The prevailing notion is that estrogenic compounds released into the environment by humans are the primary cause of the observed feminization of male fish, so the synthetic estrogen EE₂ was chosen as the focus of the experiment (Bjerregaard, 2006). Because bass species displayed a high degree of intersex characteristics and hybrid striped bass were readily available, this species was chosen as the model organism of the study. It was hypothesized that exposure of sperm cells to waterborne EDCs during the spawning process before and during fertilization might damage the cells and account for some of the observed intersex

characteristics and mass mortality in adult organisms. Deleterious effects of waterborne EDCs on sperm might also reduce the reproductive efficacy of these intersex fish. Therefore, the effects of EE₂ on hybrid striped bass sperm were chosen as the focus of the study.

Few extant studies directly examine sperm health and integrity; more often, studies feature broader physiological surveys or histologies of gonadal tissue. The lack of scientific knowledge about the directed effect of EDCs on sperm cells led to the conclusion that the study of hybrid striped-bass sperm viability post-EDC exposure would meaningfully add to knowledge in the field, which is still struggling to explain the causes of the surge in fish kills and intersex characteristics worldwide.

Therefore, the broad question “What effect do EDCs have on the reproductive efficacy of male fish?” was posed and addressed by studying two specific endpoints: (1) the percentage of hybrid striped bass sperm cells that survives direct exposure to EE₂ and (2) the amount of damage, if any, that EE₂ causes to the genetic material of hybrid striped bass sperm. Measuring the cytotoxic effects of EE₂ by examining the percentage of cells that survive direct, acute exposure to the compound sheds light on the ability of sperm cells to survive exposure in the context of spawning and fertilization. High sperm lethality during this time would have enormous impacts on the spawning fish’s reproductive efficacy. Measuring the genotoxicity of EE₂ by examining the genomic integrity of sperm cells post-exposure, sheds light on the genetic quality of the embryo. High levels of DNA damage in the spermatocyte could result in offspring with mutations, lethal or otherwise, or even in an inability to fertilize an egg. A measurable cytotoxic or

genotoxic effect of EE₂ would affect the overall reproductive efficacy of male hybrid striped bass.

To measure the two endpoints of reproductive efficacy enumerated in the research question, two assays were used: SYBR-14/propidium iodide (PI) to measure cell viability and cytotoxicity and the comet assay to measure genotoxicity. In the cell viability assay, each sperm sample, containing thousands of cells, was treated with EE₂ and stained with SYBR-14 and PI. These compounds are both nucleic acid stains, but SYBR-14 stains live cells and PI only enters cells with ruptured membranes, which are dead or dying. SYBR-14 and propidium iodide can absorb at 488 nm and emit at 518 nm (green light) and 617 nm (red light), respectively, when bound to DNA. A fluorescent-activated cell sorter (FACS) machine counted thousands of cells to survival rate of cells exposed to different treatments of EE₂. Based on a comparison of these samples, we were able to determine what, if any, cytotoxic effects the treatments of EE₂ had on the cells.

Another measure of reproductive efficacy, quantifying genetic damage to the sperm cell, was examined with the comet assay. The comet assay lyses individual sperm cells and runs single-cell electrophoresis. Each cell leaves behind a “tail” of broken or fractured genetic material. While a small tail is present in most cells (even those that are untreated), as the number of double-strand breaks in the DNA increases, smaller fragments of DNA are created, and the length of the tail increases proportional to the degree of genetic damage. To determine the degree of genetic damage, hundreds of photographs of individual sperm cells were taken. The tail lengths from each cell in all treatment groups were measured and compared across treatment groups.

Using these two assays, information was gathered on the cytotoxic and genotoxic effects of exposure to EE₂ on hybrid striped bass sperm. It was hypothesized that EE₂ exposure would significantly decrease sperm survival rate and increase observed genetic damage, and that exposure at 500 μM EE₂ would have a larger effect in both cases than at 32 μM EE₂. After analysis of the data, the comet assay did not provide data of sufficient quality to draw conclusions on the potential genotoxic effects of EE₂. However, the SYBR-14 assay demonstrated that short term exposure to EE₂ does not have a statistically significant cytotoxic effect on hybrid striped bass sperm cells at concentrations of extremely high concentrations.

Chapter 2: Literature Review

Environmental Impact

Endocrine-disrupting chemicals primarily act on the hypothalamus-pituitary-gonadal (HPG) axis which is the primary mechanism of hormonal control within the body. The HPG axis is active in animals during sexual maturation and differentiation. The HPG axis in most fish species is responsible for initiating this differentiation prior to zygote hatching. The introduction of environmental estrogens and androgens can affect the HPG axis and subsequent sexual differentiation of the animal by causing a shift away from bimodal male-female differentiation. This effect can manifest morphologically (e.g. ova-testes) or molecularly (e.g. vitellogenesis). Fish are often exposed to harmful EDCs early in life, during the larval stage of development and even before fertilization as eggs are laid by the female and fertilized externally by males in the open environment (Ankley et al., 2004). This exposure can also occur through maternal transfer of lipophilic compounds to eggs. Sexual differentiation, which naturally occurs bi-modally in vertebrates, is achieved through the production of sex hormones by the gonads during development. The two main classes of sex hormone are androgens and estrogens. These hormones are released by the gonads, which are triggered into action by two elements of the HPG Axis, the hypothalamus and the pituitary gland. During differentiation, the hypothalamus releases gonadotropin-releasing hormone (GRH). GRH controls the release of Follicle-stimulating Hormone (FSH) and Luteinizing Hormone (LH) by the pituitary gland. Both of these hormones are necessary for controlling processes such as

germ cell production and ovulation. In addition, these hormones control the synthesis of estrogen and testosterone from the gonads. Both estrogen and testosterone function in the development of sexual traits by binding their respective receptors and affecting gene transcription (Marieb, 2007).

Sexual differentiation is dependent on a delicate balance of androgens and estrogens within the body. This balance can be skewed by the presence of chemicals, such as synthetic estrogens, within the environment. Synthetic estrogens are known to mimic their natural counterparts and bind the same receptors, which forces the body to negotiate unnaturally high levels of hormone (Marieb, 2007). This alteration of natural sex hormone levels disrupts the HPG axis and thus causes a shift away from bimodal differentiation, ultimately resulting in the development of organisms with both male and female reproductive characteristics. This altered development ranges from increased production of female-specific proteins (e.g. vitellogenin), to organisms with intersex gonads, to complete sex reversal.

Endocrine disrupting compounds (EDCs) that mimic estrogens result from a number of environmental contaminants, such as certain manufacturing byproducts, effluent from wastewater treatment plants, and some pesticides (Nimrod & Benson 1996a, 1996b; Solomon & Schettler 2000; Sumpter 1998). Exposure to these estrogenic EDCs may lead to a variety of physiological problems in humans (Damstra, 2002; Guillette & Moore, 2006; Rogan & Ragan, 2007). Reports of declining sperm count in males are fairly well known (Murray, 2002). Even more striking are studies that indicate observable physical changes in human males. For example, the amount of phthalates, substances found in some plastic, in a mother's breast milk strongly correlated with

altered sexual development in their children (Lottrup, 2005). EDCs such as phthalates can be found in a large variety of substances including food containers and other household items. Additionally, pesticides, antibiotics and other potential EDCs are found in food consumed by children and constitute a major concern for children's health (Xu, 2002). In addition, phthalates and other similar chemicals have been implicated as disruptive to the endocrine systems of wildlife (Daughton and Ternes, 1999).

Even minute effects on the endocrine system can result in changes in development, reproductive ability, and behavior, especially in early developmental stages. For this reason, EDCs have an especially high impact on children. Some EDCs are persistent and lipophilic, meaning that these substances can bioaccumulate and biomagnify, resulting in higher concentrations within the animal than are present in the surrounding environment. Since EDCs are lipophilic and can bioaccumulate within fat cells, they can be transferred from parent to child while in the womb and through breastfeeding (Damstra, 2002). There is increasing concern that low-level exposure to EDCs may have adverse health impacts, particularly during fetal, neonatal, and childhood development (Suk et al., 2003). Exposure to estrogenic EDCs may lead to cryptorchidism, semen abnormalities, and hypospadias (Carlsen et al., 1993; Giusti et al., 1995; Giwercman et al., 1993; Sharpe & Skakkebaek 1993; Toppari 1996; Toppari et al., 1996).

EDCs can also impact adult humans. According to Toppari et al., "... declining semen quality has been reported from Belgium, Denmark, France, and Great Britain. Additionally, the rates of testicular cancer, hypospadias and cryptorchidism have increased during the same time span (Toppari et al., 1996). Research suggests that the

adverse changes may originate in fetal life or childhood (Biggsy et al., 1999). Exposure of the male fetus to supernormal levels of estrogens can result in the above-mentioned reproductive defects (Ibid). In adult women, EDC exposure has been linked to the occurrence of endometriosis, a condition where tissue naturally found in the uterus grows in other areas of the body. This condition can cause pain in addition to creating reproductive concerns (Mayani, 1997).

In several fish species that have been exposed to environmental estrogens, researchers have documented the formation of ova-testes and testes-ova (Ibid). In England, fishermen have observed this phenomenon in roach caught downstream of a Wastewater Treatment Works (WWTW) in London for the past quarter century (Tyler & Jobling, 2008). In a field study done on roach populations across England, a positive correlation was drawn between concentration of effluent in water and percentage of wild intersex roach. Scientists observed that roach collected from points downstream of multiple WWTW sites were between 16% and 100% intersex, compared to between 11% and 44% of roach collected from upstream sites (Jobling et al., 1998). At the sites themselves, scientists recorded an intersex rate of 86% (Jobling et al., 2006). Because the effluent was known to contain substances of high estrogenic activity (E_1 , E_2 and synthetic EE_2 , which altogether accounted for 80% of estrogens present in obtained water samples), the scientists connected environmental estrogenic activity to endocrine disruption and the development of intersex characteristics in fish. Similarly, in Denmark, Bjerregaard et al. correlated the presence of intersex fish with the presence of estrogenic sewage effluent in water. The scientists sampled five fish populations. Two of the five populations were taken from effluent-free sites—Lakes Almind and Ravn. The

remaining three fish populations were taken from effluent-affected sites—Egaa, Aarhus Brook, and Kristup Landkanal. The scientists' findings were similar to those of Jobling (2008). They found that there was the presence of intersex gonads at all sites--including the sewage-unaffected sites, which had an average intersex rate of 4.8% (Bjerregard et al., 2006). However, this rate was concluded to be significantly lower than the intersex rates of the fish taken from affected sites (Aarhus Brook, Egaa, and Kristrup Landkanal), which were 6.7%, 10.6% and 26.5%, respectively (Ibid). Additionally, the scientists found significant differences in the *severity* of intersex across the five populations. They quantified severity by assigning numbers (0-5) to the number of testicular oocytes (TO) counted in cross-sections of male gonadal tissue (0=normal tissue, 5=tissue with >30 oocytes). Among fish taken from Almind and Ravn, the intersex severity numbers were 0.2 and 0.55, respectively. However, among fish taken from AB, Egaa, and KL, the severity numbers were 1.9, 1.0, and 0.6, respectively. The data from AB and Egaa represent a significant difference between frequency and severity of intersex among the two groups (affected and unaffected) of fish, and caused the scientists to again link the presence of environmental estrogens to the occurrence of intersex characteristics.

In the United States, scientists produced similar findings in a study that connected human use of land (for sewage purposes, agricultural purposes, etc.) to the prevalence of intersex characteristics in smallmouth bass. Blazer sampled viable numbers of fish from three different areas in and around the Potomac River water basin: the Potomac's South Branch, which has a low population density and a high percentage of farmed land; the Shenandoah Valley area, which has a high population density and a high percentage of farmed land; and an out of Basin area, which has a low population density and low

percentage of farmed land. The scientists found that the presence of intersex characteristics, specifically testicular oocytes, was highest in areas of high anthropogenic use. The Shenandoah Valley collection area, which spanned Page, Shenandoah, and Warren counties, produced fish with intersex rates of 100%, 80% and 100%, respectively. Of the areas sampled by the researchers, this particular area had the highest percentage of arable land (38.325%); specifically, it had the highest numbers of poultry and cattle according to data collected by the USDA in 2004 (Blazer 2005). Because the area also had the highest human population density according to the 2002 census (35.5 persons/km²), researchers were able to link heavy human use of the land to a significant occurrence of TO in collected specimen.

These findings were further supported by the researchers' additional findings. Just as researchers connected high TO levels to high land use, so too did researchers discover that sample sites outside of the Potomac basin yielded lower percentages of fish with intersex characteristics. Whereas the Shenandoah sites yielded intersex rates of up to 100%, the out-of-basin sites yielded a median intersex rate of only 36%. For the most part, these sites had significantly lower human and livestock population densities when compared to the Shenandoah site. However, even within the out-of-Basin group, there existed variation that illuminated, yet again, the significance of anthropogenic impact on endocrine disruption. For example, even in the out-of-basin group, there was one site, Greenbrier, that exhibited an above average prevalence of TO which corresponded with a relatively high percentage of agricultural use. The percentage of TO observed in fish at the Greenbrier site was 75%, a full 39% higher than the median for all fish sampled from out-of-basin sites.

The scientists found additional evidence to support the significance of agricultural runoff in the development of abnormal gonads and endocrinology in fish. Researchers found that fish that were sampled from the Potomac's South Branch, a third sampling location, yielded intermediate levels of endocrine disruption reflective of a combination of low human population with high agricultural land use. On average, the South Branch sites had a population density of 8.5 persons/km²—exactly the same as the out-of-basin sites, yet 27 fewer persons/km² than the Shenandoah sites. This site, similar to the high-intersex Shenandoah sites in agricultural land use, yet dissimilar to the Shenandoah sites in its population size, yielded an average intersex rate of 60%—an intermediary between the Shenandoah's 93%, and the 36% of the out-of-basin sites. This is significant because it shows the true spectrum of the chemicals responsible for causing endocrine disruption. In combination, these results argue strongly for a direct correlation between endocrine disruption and degree of human land use.

Although the literature agrees on the correlation between anthropogenic use with increased amounts of endocrine disruption (e.g. TO), it is important to examine what this disruption actually means for fish populations. Specifically, research has not sufficiently explored the extent to which endocrine disruption influences population stability and ecosystem dynamics.

In order to understand the influences of endocrine disruption on these two factors, Jobling et al (2002a) compared the reproductive quality of male and female roach downstream of wastewater treatment plants with that of unexposed roach at three reference sites. Researchers ultimately concluded that EDCs were jeopardizing the functioning of both male and female reproductive systems. One of the markers they

looked at was production of sperm. They discovered that, among the fish populations taken from the polluted Aire and Nene sites, individuals produced less milt than individuals at the reference sites. Specifically, at the Aire and Nene sites, only 50% and 57% of male fish, respectively, produced sperm at all, compared to 100% of male fish at the reference sites. Of the male fish that were able to produce sperm, Aire and Nene individuals produced less sperm than their unexposed counterparts (0.2 and 1.0 million sperm/gram body weight, respectively, compared to 1.7 million sperm/gram body weight in reference site fish). Additionally, male fish from the Aire site were determined to have a statistically significant decrease in sperm cell density when compared to reference site fish (2×10^6 sperm/ μ l milt compared to 4.2×10^6 sperm/ μ l) (Ibid).

In addition to the findings regarding milt quality, Jobling et al. found that sperm cells in exposed fish were at earlier stages of development than sperm cells of unexposed fish. Both spermatogonia A and spermatocyte B are intermediaries in the spermatogenesis process. Concentrations of these intermediaries across cells can indicate an organism's specific stage of spermatogenesis and testicular development. Unexposed fish, which were determined to be in early stages of mitosis/ the late stages of meiosis, yielded sperm intermediary percentages of 96.2 (spermatogonia A) and 3.75 (spermatocyte B). In contrast, exposed fish yielded respective numbers of ~86.0 and 2.6 – a statistically significant difference. The discrepancy between the exposed and unexposed sperm population make-up suggests developmental delays in the exposed fish. These delays could have significant effects on fish fecundity, given the time-sensitive nature of the spawning process and need for sperm to be fully developed for successful egg fertilization.

Furthermore, histological findings were used to assess the effects of the EDCs on experimental fish. Researchers observed that 8.3% to 38% of fish at the Nene and Aire sites had underdeveloped testicular lobes, which again signified delayed development. They also observed the absence of reproductive ducts in some fish, and the presence of ducts that ended before the genital opening, causing expulsion of milt into the abdomen, in others. Both of these findings were associated with the 43% to 50% of fish that were unable to spermiate. Reference fish did not display these defects. The inability to spermiate results in lowered reproductive viability, thus providing a link between EDCs and decreased individual and population fecundity. These histological findings were consistent with the sperm-based findings, confirming the existence of EDC-related abnormalities and allowing the researchers to hypothesize potential adverse effects on fish fecundity. Jobling et al. strongly suggested that the presence of these chemicals compromises the reproductive efficacy of the roach population. Further, they also were unable to link their findings to larger scale ecosystem effects. Due to the variability of environmental factors that could have influenced the experiment, Jobling et al were unable to conclude causality. These factors include, but are not limited to temperature, food availability and competition, which all can affect the maturity of sperm collected from different lakes.

In a subsequent experiment, the group focused on the effect of EDC exposure on fecundity. Jobling et al collected male and female gametes from individuals at two sites-- the Nene River (as before), and a reference site. To measure whether exposure had had an effect on fertility, researchers performed the following crosses: a) unexposed male x unexposed female (reference site x reference site), b) unexposed male x exposed female,

c) exposed male x exposed female. The results, consistent to the earlier hypothesis that EDCs affect fecundity, prove a proportionally inverse relationship between EDCs and reproductive success. They found that there was a 93% fertilization rate when the gametes of two unexposed fish were crossed. There was a comparably high fertilization rate, 89%, when reference site males were crossed with exposed females from the Nene site. However, when exposed males were crossed with exposed females the fertilization rate dropped to 68%, indicating significant damage to reproductive efficacy. Furthermore, it was found that the degree of exposure and feminization of male fish was inversely proportional to the male's chance of being reproductively successful as measured by the production of offspring. For example, in fish that were mildly feminized, the chances of successful fertilization, progression to eye development, and hatching were roughly 80, 60 and 50%, respectively. But in severely feminized fish, these respective chances were roughly 20, 18 and 15%. Because fecundity is dependent on both the quality and quantity of sperm, the experimental results also speak to the hypotheses that were made in the first experiment, which centered on EDC-damaged sperm, ducts, and gonads acting together to reduce fertility.

These findings are particularly significant when considered in the contexts of fish spawning, a delicately balanced process. Spawning typically involves multiple male fish vying for the eggs of one female. Quality and quantity of sperm is especially important—the more sperm a fish has and the faster the sperm swim, the greater chance that fish has of successfully breeding. Though the researchers could not make a statistically significant connection between sperm motility and reduced fecundity, they did link reduced sperm motility to increased feminization, so it is possible that the latter

affects the former. Assuming this possibility, the entire fish population could face a severe decline in numbers should a significant portion of its members suffer from EDC exposure. This possibility was tested and realized in a multi-generational experiment which exposed fathead minnows to low-dose EE₂ exposure over generations. The experiment resulted in complete population collapse within 3 generations, demonstrating the significant dangers posed by EDC exposure to fish population (Kidd et al., 2007). It is significant to note that the low 2ng/L exposure is potentially lower than EDC concentrations in environmental conditions. Furthermore, a significant fish population decline in a natural lake would not just affect that specific fish population, but would affect the entire ecosystem of which it is a part (Jobling et al. 2002b). Large scale fish kills in the Potomac and Shenandoah River watersheds are illustrative of the widespread effects population decline has on ecosystems.

Basic Endocrinology

The process responsible for intersex development is called endocrine disruption, which works through the endocrine system. All the tissues in the body are coordinated by chemical messengers such as neurotransmitters, neuroendocrine hormones, paracines, autocrines, and endocrine hormones. The chemical messengers relevant to this study are endocrine hormones. Glands and certain cells release endocrine hormones into the bloodstream. The glands and cells that release these hormones include reproductive tissues (ovaries and testes), hypothalamus, pineal gland, pituitary glands, thyroid, parathyroid, thymus, pancreas, kidneys, and the adrenal gland. Endocrine hormones are

essentially long distance messengers that travel via the bloodstream. When endocrine hormones are released, they bind to their specific receptor to initiate reactions. These receptors can be located on the outer membrane, in the cytoplasm of the target cell, or on the cell's nuclear membrane. Once endocrine hormones bind to receptors, they initiate transcription, by causing a cascade of chemical reactions. These reactions alter the local and/or global physiological environment (Guyton & Hall, 2005).

There are three classes of endocrine hormones: protein/polypeptides, steroids and derivatives of amino acids, such as tyrosine. Estrogens are steroids. Steroids are synthesized physiologically in many tissues, including the adrenal cortex, and the gonads. They are derived from cholesterol and are highly lipid soluble. In response to certain cell signals, steroids are then released into the interstitial fluid where they are then carried off by the blood. In the blood, steroids are bound to plasma proteins; at any given time, less than ten percent of total steroids float free in blood (Guyton & Hall, 2005).

The concentration of steroids in the blood is closely controlled via negative feedback. This control is often related to the activity of the target tissue and not the actual secretion rate. Additionally, the action of the hormone is controlled by the number and sensitivity of the receptors. Controlling the amount of hormone secretion is important because too much hormone can cause receptor proteins to malfunction. For example, excess hormone presence can cause the inactivation of a receptor or signaling molecule, the isolation of the receptor, decreased receptor production, and even the destruction of the receptor (Guyton & Hall, 2005).

The rate of clearance of endocrine steroids from the blood is called the metabolic clearance rate, or rate of removal. This rate is measured in terms of the rate of the disappearance of the hormone compared to the concentration of the hormone in each mL of blood plasma. The hormone can be cleared in a few ways: by the metabolic breakdown of the hormone, by binding with tissue, and by excretion from the body through either bile or urine. When bound to plasma proteins, steroids are cleared at a very slow rate and can stay in the blood for several hours or even days (Guyton & Hall, 2005).

Endocrine Disrupting Compounds

EDCs include a wide variety of chemicals, most of which are introduced to the ecosystem by humans (Oregon Environmental Quality Website, 2008). EDCs are grouped together by common effects, not by basis of composition (Kortenkamp, 2007). It is thought that fire retardants and agricultural run-off, including some pesticides, and hormones in animal waste, are major sources for EDCs (Rosen et al., 2006). It is important to note that EDCs may be additive; that is, effects are cumulative even if the individual chemicals differ (Brian et al., 2005). Originally, waste-water treatment plants were thought to be the primary source of EDCs entering the water. This theory, however, was put into question by the discovery of intersex fish found upstream of these plants, in 'pristine' areas such as the South Branch of the Potomac, indicating EDCs may also come from agricultural chemicals and runoff (Chambers, 2006).

Estrogens and other hormones can also function as EDCs. Estrogenic EDCs occur naturally and are not necessarily harmful. However, estrogenic EDCs can have negative

environmental effects, especially when they are artificially introduced either to a new environment or in new quantities to an environment where the compound already exists. Estrogens are released by various glands and influence bodily activity by binding to receptors to initiate reactions. Problems occur when foreign estrogens or artificially high levels of estrogens enter the body and upset the body's equilibrium. Levels of estrogens in the body are naturally very low, meaning that when the levels shift by even small concentrations the effects can be dramatic (Guyton & Hall, 2005). Human involvement continues to contribute to the presence of EDCs in new environments and increased levels of EDCs in environments where the compounds already exist.

17- α -ethynylestradiol (EE₂)

Generally speaking, diverse arrays of compounds found in aquatic ecosystems are known to affect the endocrine systems of aquatic animals (Jobling et al., 1995). Though these

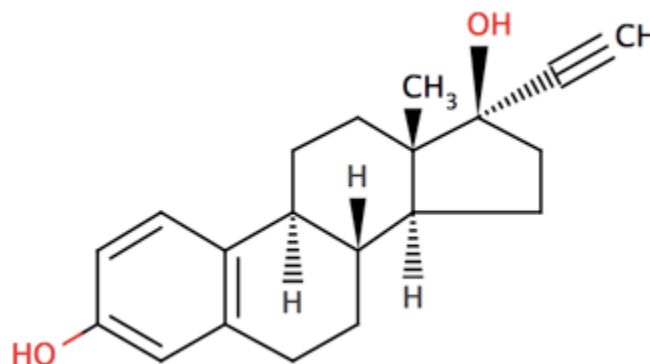


Figure 1: Structure of EE₂.

compounds are known to be structurally diverse, there has been a distinct concentration on compounds with estrogenic activity due to its pronounced effect on primary and secondary sex characteristic development (Ibid)). 17-alpha-ethynylestradiol (EE₂) (Figure 1), the synthetic estrogen in birth control pills can alter balance of hormones as do other EDCs. Steroidal estrogens in female animals, known as estrone (E₁), estradiol (E₂), and estriol (E₃), are produced in the ovaries, via the enzymatic process of aromatization of

androstendione, an androgenic hormone. The androgenic hormone testosterone often occurs in female fish as levels equal to those in males; it is the ratio of estrogen to testosterone that determines functionality. Synthesis of these compounds occurs in response to the release of two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), by the gonadotropes of the anterior pituitary gland (Whitehead & Nussey, 2001). EE₂ competes with E₂ (17-beta-estradiol), the naturally occurring estrogen, to bind to two estrogen receptor (ER) subtypes on the nuclear surface, known as α and β , which are encoded by two separate genes (ESR1 on chromosome 6 and ESR2a on chromosome 14 respectively). Once EE₂ binds to its receptor, it can enter the cell in a receptor-ligand complex, which subsequently forms a homodimer. At this point, the dimer can then enter the nucleus of the cell and act as a transcription factor for a number of genes. For example, EE₂ has been shown to down-regulate nuclear excision repair genes in studies with *Danio rerio* (Notch, 2009).

A primary role of the natural estrogen E₂ in the liver of adult female fish is to activate the synthesis of specific gene transcripts that encode proteins required for reproduction by binding to the estrogen receptor (Purdom et al., 1994). Several genes known to be activated by this process include those that encode the ER itself, vitellogenins, and choriogenins (Arukwe et al., 2001; Bowman et al., 2000; Celius et al., 2000; Denslow et al., 2001a, 2001b; Flouriot et al., 1995, 1996, 1997; Folmar et al., 2000; Funkenstein et al., 2000; Hemmer et al., 2001; Lattier et al., 2001; Le Guellec et al., 1988; Lim et al., 1991). However, in males, the normal endogenous levels of E₂ are sufficient to induce only very small amounts of plasma vitellogenins and choriogenins (Arukwe et al., 2001; Copeland et al., 1986). When males are exposed to natural or

anthropogenic estrogens, which can either enhance the steady-state concentrations of endogenous E₂ or bind directly to the ER, the result is an increase in the circulating levels of vitellogenin and choriogenin proteins. Vitellogenin and choriogenin synthesis in male fish have therefore become accepted assays for measuring exposure to estrogenic chemicals (Arukwe et al., 1997; Bevans et al., 1996; Celius et al., 1999; Celius & Walther, 1998; Denslow et al., 1996; Folmar et al., 1996, 2000; Hemmer et al., 2001; Jobling et al., 1995).

A field study which measured the androgen/estrogen levels in carp has also suggested a correlation between exposure to estrogenic compounds and a shift in plasma androgen and estrogen levels, indicating estrogenic activity (Folmar et al., 1996). Another field study focused on the English roach population suggested a correlation between sewage effluent exposure and intersex characteristics (Routledge, et al., 1998). In lab studies, injected estrogenic substances resulted in the presence of intersex gonads in medaka and carp (Gimeno et al., 1998). Lab studies have also confirmed the stunted growth of testes in male rainbow trout exposed to endocrine disruptors (Jobling et al., 1996). Therefore, these estrogenic substances are understood to significantly affect sexual differentiation in fish.

17- α -ethynylestradiol is considered to be the most potent estrogenic compound present in the environment (Scholz & Gutzeit, 2000). The chemical, which is used in the formation of oral hormonal contraceptives, is the base for one of the most prescribed medications in the world (UN Population Division, 2006). After being metabolized in the liver and gut, EE₂ is excreted in human urine and fecal matter, and is therefore present

in sewage effluent in concentrations up to 7 ng/L (Ibid). Due to its high potency and environmental relevancy, EE₂ is of utmost research interest.

EE₂ and Fish

In controlled lab studies that measured effects of EE₂ on medaka survival rate, XX/XY sex ratio, gonadal growth, spawning, sexual differentiation, gonadal histology, and aromatase gene expression, researchers found that XY medaka exposed to the highest concentration of EE₂ (100 ng/L) developed into females (Scholz & Gutzeit, 2000). The experimental fish were juvenile medaka and were exposed to concentrations of 1, 10 and 100 ng/L of EE₂ by bath over a period of two months. The high concentration were considered female because they lacked male-specific coloring (red chromatophores), and because histological examination revealed the presence of female gonads. In the same study, researchers found that with increasing EE₂ exposure, there was a proportional decline in the daily number of eggs spawned by each female as well as a decline in the percentage of females producing any eggs at all (Ibid). At the highest concentration (100 ng/L), none of the female fish were egg producers (Ibid). In terms of gonadal histology, males exposed to the less potent 1 and 10 µg/L concentrations did not exhibit gonadal differences when compared to the control (testicular lobes were intact). However, in XY females exposed to the 100 ng/L concentration, histological examination revealed the presence of immature ovaries at various points in follicular development, indicating that the EE₂ had an impact on sexual differentiation (Ibid). On the molecular level, aromatase, the enzyme responsible for the production of estrogens from androgens,

was found to be present in the testis of males exposed to the 10 ng/L concentration. XY females had the highest aromatase gene expression; these individuals were also exposed to the highest levels of EE₂ (Ibid). The results confirmed that positive molecular changes are not always mirrored by anatomical changes, as no ova-testis or testis-ova were observed (Ibid). Scholz posits that the 6 week recovery period following exposure could have been responsible for this.

In a study which measured the effects of environmentally relevant concentrations of EE₂ on newly spawned Chinese rare minnows, sex ratio findings were similar to those reported by Scholz. At 180 days post hatch, no phenotypic males were identified at the 1 and 4 ng/L concentrations (Zha et al., 2008). Gonadal histological analysis confirmed a bias toward female genital tissue, with this bias increasing in a dose dependent manner (ratios were 30:48, 14:57, and 0:30 at 0.2, 1, and 4 ng/L, respectively) (Ibid). Researchers found that the fish exposed to EE₂ at 1 and 4 ng/L had significantly deformed ovarian tissue when compared to the control (Ibid). Another significant finding was the presence of oocytes in the seminal vesicles of genotypic males exposed to 0.2 and 1 ng/L of EE₂ at rates of 22% and 64%, respectively (Ibid). There were also documented differences in fish body length and weight (compared to the control) at the 4, 16, and 64 ng/L concentrations, suggesting that the EE₂ had developmental effects on these individuals.

Perhaps most compellingly, Zha et al. documented significant fish mortality in the 16 and 64 ng/L groups (100% by 120 dph), 76% mortality in the 4 ng/L group, and <40% mortality in the 1, 0.2 and 0 ng/L groups, suggesting that EE₂ acted in a dose dependent manner with severity to disrupt the experimental population to result in fatality (Zha et al.,

2008). Scientists also found that only fish in the 1, 0.2, and 0 ng/L groups were able to reproduce, suggesting that EE₂ had a negative impact on fecundity. Inferring from these results, researchers determined a positive correlation between EE₂ exposure and sex ratio shift, gonadal abnormalities, the presence of ova-testes, decreased body length and weight, higher fish mortality, and lower fish fecundity. These results suggest that EE₂ in the environmental could have these same deleterious effects on individuals, affecting the health and sizes of entire fish populations (Ibid).

EE₂ and Mammals

Importantly, the negative effects of EE₂ have not only been confined to fish populations. Studies have also investigated the effects of EE₂ on mammalian species. In 2008, researchers exposed a population of rats to EE₂ in drinking water and found that rats exposed to 10 ng EE₂/L experienced a decrease in litter size (Vosges et al., 2008). Whereas the control group (of 27 litters) experienced no small litters, the group exposed to EE₂ had a small birth rate of 6:24 (Ibid). Moreover, after the 3-week exposure period, EE₂-exposed rats were growing at a rate 40% slower than those of the control group (Ibid). Vosges notes the similarity of these findings to those of Goyal et al., which reported a 50% occurrence of small size litters, as well as size decreases, in rats exposed to the estrogenic compound Diethylstilbestrol (DES) (Ibid). The findings of Goyal and Vosges indicate that, if exposure to EE₂ and other EDCs causes reproductive harm to mammals as well as fish, human exposure to these compounds could easily produce deleterious effects in human populations. Human exposure has been already been

scientifically associated with the occurrence of female and male reproductive abnormalities (e.g. endometriosis, declining sperm count), as well as with non-reproductive abnormalities (e.g. liver damage, ADHD) (Vosges et al., 2008).

Sperm

Sperm cells function the same across animal species. If a chemical affects fish sperm, it could also affect human sperm in a similar way. The fact that human and fish sperm are similar contributed to the reason for using fish sperm as a measure of reproductive efficacy.

Estrogen receptor (ER) expression is activated by the presence of estradiol in a cell. ER's main function is regulating gene expression but also has different functions such as signaling to G proteins via blood plasma membrane (Levin, 2005). ER expression, in particular ER- α is found in mammal spermatocytes and spermatids (Pelletier et al., 2000; Pentikainen et al., 2000; Durkee et al., 1998). ER- α expression, and also ER- β expression, are found in spermatocytes and spermatids of mature fish sperm cells as well (Wu et al., 2001). Wu et al. reports the first instance of ER expression found in the sperm cells of nonmammalian species, illustrating even more similarity between human sperm and fish sperm. The role of ER- α and ER- β in fish sperm is not fully understood. It has been suggested that ERs are related to motility, and immotile sperm could indicate infertility (Ibid). This is supported by a study showing that male mice with an ER alpha deficiency are infertile (Eddy et al., 1996). In the study, male mice that were

homozygous for an ER gene mutation were all infertile, which indicates that ER play a vital part in fish reproduction.

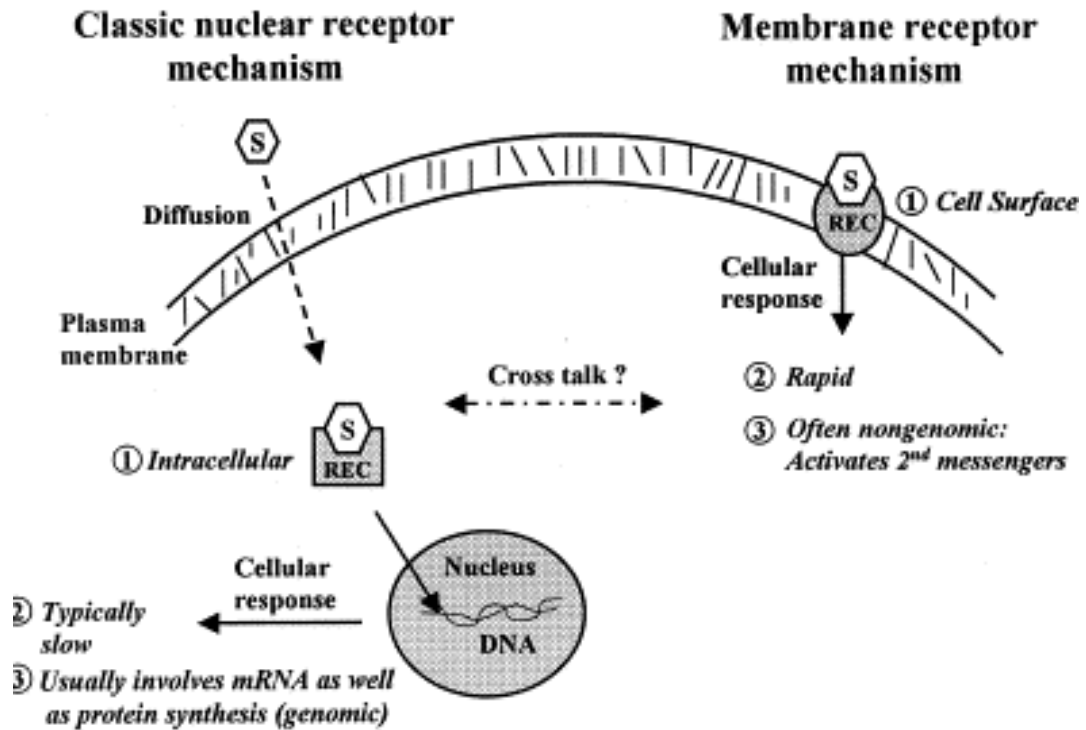


Figure 2: Characteristics of steroid hormone action. The figure shows nuclear and membrane receptor-mediated mechanisms of steroid hormone action. Numbers refer to defining characteristics of the two steroid mechanisms (Thomas et al., 2000).

Two forms of receptor initiated cascades are classic nuclear and membrane receptor mechanisms (Figure 2). The classic nuclear receptor mechanism allows steroids (such as estrogens) to diffuse across the plasma membrane where the receptor takes it to the nucleus (Thomas et al., 2000). With the membrane receptor mechanism, the receptor is bound to the cellular membrane (Ibid). Evidence of membrane receptor mechanism has been found in the testes and sperm cells of Atlantic croaker (*Micropogonias undulatus*), adding to the complexity of where these steroid hormones can potentially act. This indicates that EE₂ has more ways of entering the cell and causing damage to the DNA.

The integrity of DNA in sperm is extremely important. DNA can be damaged in a variety of ways, an example being freezing and thawing sperm causing DNA fragmentation (Zilli et al., 2003). DNA damage can severely impact fecundity, a study by Fauvel et al. (1998) shows that decreased sperm motility and a lower hatch rate of eggs fertilized by DNA-damaged sperm.

EE₂ can also cause aneuploidy, an abnormal number of chromosomes, in sperm cells. Semen samples from male rainbow trout that were exposed to EE₂ for fifty days and sperm aneuploidy levels were then measured using fluorescence showed development of aneuploidy in sperm cells. In addition, aneuploidy in sperm cells was shown to cause aneuploidy in embryonic cells which ultimately results in lowered reproductive success (Brown et al., 2008). The potential of genetic disturbance and/or cellular toxicity in sperm cells, particularly by EE₂, is very great and is why the effects of EE₂ on fish sperm should be explored.

Hybrid striped bass were chosen as the fish to obtain sperm from due to their availability. Hybrid striped bass spermiate during spring, which was when the experiment took place. Hybrid striped bass are also a large fish, enabling large quantities of sperm to be obtained without harm to the fish. These factors allow the best chances of using fresh sperm for experimentation. Frozen sperm were avoided as freezing sperm can damage the DNA of the sperm cell (Zilli et al., 2003). A study in 1998 researching the rate of egg insemination by frozen sea bass sperm suggests that freezing sperm results in decreased fertilization ability (Fauvel et al., 1998). Since damaged DNA, as indicated by strand breaks, correlates inversely to reproductive efficacy, it is very important to use fresh sperm when possible.

Dimethylsulfoxide (DMSO), is a commonly used lipophilic solvent. DMSO easily permeates membranes to dilute fish sperm when cryopreserving the cells (Taitson, 2008; Vivieros, 2009). Caminada, Escher, and Fent used DMSO as a solvent to test the *in vitro* cytotoxicity of 34 pharmaceutical compounds on two fish cell lines. Using a maximum DMSO concentration of 2%, their study was able to detect significant cytotoxicity in 21 of their tested compounds (Caminada, Escher, & Fent, 2006). In 2000, researchers used DMSO as a solvent in a study that examined the DNA of a bush cricket (Simmons & Achmann, 2000). This indicates that DMSO is safe enough to use when measuring the damage of DNA.

Plasma membranes have been found to be much better protected with increasing DMSO concentration. Specifically, 10% DMSO shows the highest percentage of sperm with plasma membranes intact. Glycine has not been shown to have an effect on plasma membrane integrity. He and Woods demonstrated the ability to maintain a high level of cell viability and plasma membrane integrity, despite cryopreservation in striped bass sperm, using DMSO (He & Woods, 2004).

General sperm motility and viability in Atlantic cod and haddock after DMSO cryopreservation after 90 days of storage is not different from fresh (DeGraaf & Berlinsky, 2004). Additionally, there is no significant difference in fertilization rates between fresh and cryopreserved sperm samples taken from the same male or among males. These findings were produced from research that collected and assessed sperm motility and viability during an 8-week spawning period. Sperm cryopreserved in modified Mounib's extender, with DMSO, had the highest post-thaw motility and cell viability. The study demonstrated that acceptable post-thaw cell viability, motility, and fertilization rates could be obtained from cryopreserved sperm (Ibid).

However, Lahnsteiner et al.'s 1992 and 1996 findings contrasted with the above findings of Achmann, Simmons, He, Woods, DeGraaf, and Berlinsky. Lahnsteiner used DMSO to cryopreserve sperm from grayling. After thawing, researchers used SYBR-14 and PI fluorescent stains to assess the morphological damage and sperm quality. They found a marked decrease in sperm quality: 40%–50% of all sperm were completely damaged, 30%–40% were altered to a measurable degree, and only 10%–20% remained wholly unaffected (Lahnsteiner et al., 1992). Additionally, Lahnsteiner found that cryopreserved rainbow trout spermatozoa assessed using SYBR-14 and PI fluorescent stains sustained notable morphological damages. Decreases in sperm quality of these individuals matched studies of grayling sperm: 40%–50% of all sperm were completely damaged, 30%–40% were altered, and 10%–20% were intact (Lahnsteiner et al., 1996). Though the two Lahnsteiner studies suggest that DMSO is not an ideal solvent for sperm studies, it is important to note that Lahnsteiner et al.'s findings contradict commonly accepted standards in the field, which are consistent with the studies of Achmann, Simmons, He, Woods, DeGraaf, and Berlinsky. He and Woods, who show that DMSO-cryopreserved sperm cells have levels of motility and membrane integrity that make them suitable for experimental use, explain that any DMSO-induced DNA damages can be accounted for by using a DMSO control (He & Woods, 2004).

Comet

Another measure of sperm quality and reproductive efficacy is the comet assay, or single cell gel electrophoresis. The assay measures DNA damage in individual cells in

terms of double stranded DNA strand breaks, thus sensitively determining the level of genotoxicity in a particular group of cells (Lee & Steinheart, 2003). The comet assay can be used as a quantitative measure of DNA strand breaks. The original comet assay experiment, established by Singh, et al. in 1988, used human lymphocytes and induced DNA damage through graded amounts of x-irradiation or treatment with peroxide. Single cell microgel electrophoresis was subsequently used to measure damage (in terms of DNA strand breaks) under alkaline conditions. The assay was also used to detect the degree of DNA repair in cells that were allowed to incubate after x-ray or peroxide exposure. The study concluded that the comet assay is an extremely sensitive method for detecting DNA damage and repair. The test is commonly used for DNA damage quantification, as well as the use of peroxide as a positive control for DNA damage (Singh, 1988).

The comet assay is most commonly used as a quantification of DNA damage in somatic cells (Morris et al., 2002). Recently in the 1990s, it has been used on human sperm cells and has contributed to research concerning sperm viability and *in vitro* fertilization (Lee & Steinheart, 2003). In addition, the method has been used as a reliable measure of DNA damage in cryopreserved sperm. A comparison of the reliability of comet assay and neutral gel electrophoresis in measuring DNA damage in fresh human sperm to cryopreserved sperm (liquid nitrogen) found the comet assay to be reliable with cryopreserved samples (Duty et al., 2002). For preservation of gamete cells, four conversion methods of freezing have been compared: flash freezing with cryopreservative, flash freezing without cryopreservative, programmable freezing with cryopreservative, programmable freezing without cryopreservative. Flash freezing

without cryopreservative of human semen yielded the highest correlation to fresh human semen ($R=0.88$), suggesting that for at least some types of cells, DNA damage to gamete cells during cryopreservation is not necessarily substantial enough to invalidate results. Comet results for DNA damage in cryopreserved sperm are fairly reliable; however, the use of certain cryoprotectants may correlate with less accurate results (Duty, 2002).

The assay has been applied previously to testing the effects of EE₂ on gametes and embryos. The assay was used to examine the genotoxic and embryotoxic effects of three pollutants as treatment on oyster gametes. Benzo[a]pyrene (BaP), the synthetic estrogenic hormone 17- α -ethynylestradiol (EE₂), and the organochlorine pesticide endosulfan (ES) were chosen as the potentially relevant chemicals. Exposure of the gametes (in dilutions of sea water) to each chemical was monitored, and resulting larvae were assessed via comet assay for DNA strand breakage (Wessel et al., 2007). EE₂ displayed no toxic effect on oyster embryos for the concentrations that were applied (from 0.02 to 1.7 nM). (Wessel et al., 2007). Results suggest low levels of EE₂ are not sufficient to produce noticeable DNA damage, but do not preclude the possibility of damage at higher exposure concentrations. Similarly, no abnormalities in larval physiology were observed after EE₂ treatments. It is still unclear whether the development of secondary sexual characteristics and behaviors will remain unaltered upon reaching sexual maturity.

Previously, Liney, et al. exposed roach fish, to various concentrations of wastewater effluents and found a significant correlation between concentration and single-strand DNA breaks in blood cells and gill cells as measured by the comet assay (Liney et al., 2006). In a similar experiment performed by Filby et al., fathead minnows were exposed to three types of treatments: sewage effluent only, effluent and EE₂, and

EE₂ only (Filby et al., 2007). The effluent exposure inflicted genotoxic damage when exposed to both blood and gonadal cells. However, only the males exhibited genotoxic damage in gonadal tissue when exposed to EE₂ alone. So far in the literature, many studies have shown the genotoxic effects of EE₂ and other EDCs in somatic cells. However, very little experimentation has been done to look directly at genetic damage in the gametes. To be reproductively viable, sperm cells must possess intact genomic DNA. If the DNA integrity is compromised, then the population would be at risk of genetically abnormal offspring.

SYBR-14/PI

The SYBR-14 stain is used as a live/dead assay and is a dye which will only be taken up by active transport, an activity of living cells that is lost upon death. It is a fluorescent staining assay commonly used for assessing cytotoxicity with Dimethylsulfoxide (DMSO) cryopreserved striped bass sperm. Studies have investigated DMSO and glycine as they relate to plasma membrane integrity and mitochondrial function (two classic indicators of cell viability) in cryopreserved striped bass (*Morone saxatilis*) sperm. SYBR-14 and propidium iodide (PI) fluorescent stains in conjunction with microscopic examination are used to assess the plasma membranes of cells. SYBR-14 is a membrane-permeant nucleic acid stain that labels live sperm with green fluorescence. PI can be used concurrently with SYBR-14, since it is membrane-impermeant propidium iodide; it serves to label the nucleic acids of only those sperm with compromised membranes with red fluorescence (Moore et al., 1998).

Chapter 3: Methodology

Preparation of Sperm

Sperm samples were collected the day of experimentation from a total of 9 different fish by 3 group members over two experimental days. Hybrid striped bass (*Morone chrysops x Morone saxatilis*) were used as the source of semen for our analysis. The fish were raised in 500 gallon 6 foot diameter circular tanks on a diet of Zeigler Brother "Gold" slow sinking Hybrid diet (42% protein, 16% fat and 3% fiber). Fish were held under a computer-controlled (Graphic-Eye, Inc.) photo-thermal program in our indoor laboratory that simulated the photoperiod for the Maryland latitude. They began to spermiate when water temperature reached 15°C.

An anesthetic bath was prepared using tricaine methanesulfonate (MS-222) at a dosage of 100 mg per liter of water. A spermiating, male hybrid striped bass was selected opportunistically and placed in the anesthetic solution until sedated. The bass used were full siblings reared in the same laboratory environment and presumed to be of equal health and quality. The sedation process was carefully monitored to prevent drug overdose. Sedation was deemed sufficient by observation of a slowing of opercular movement and stillness upon lifting from the water. The fish was gently lifted, belly-side up from the bath. Sperm was dispensed by stroking the fish's upturned belly from anterior to posterior end with constant pressure. The extracted sperm was collected from the fish's urogenital opening and dispensed into a 1.5mL Eppendorf tube on ice. This

process was repeated for two additional fish yielding 3 fish per experimental trial, and thus a total of 9 fish in 3 experimental trials.

The sperm concentrations were determined using a Mackler chamber. Serial dilutions with isotonic Hanks Balanced Salt Solution (HBSS) were performed on the sperm samples to a working concentration of approximately 1×10^6 cells/mL. Each sample was initially diluted 1:1000 and two subsequent 1:2 dilutions were performed for a total dilution of 1:4000. The approximate concentration of each sample was affirmed using the Mackler chamber. Samples were kept on ice throughout the procedure.

Exposure to EE₂

For each fish, 1.2 mL diluted sperm sample at 1×10^6 cells/mL was aliquotted into each of five 2-mL Eppendorf tubes on ice. The samples were assigned to one of five experimental groups: no treatment, 5% DMSO, 32 μ M EE₂ in DMSO, and 500 μ M EE₂ in DMSO, and positive control (see Table 1). No additional solutions were added to the sperm samples in the “no treatment” group. Hydrogen peroxide (H₂O₂) was added to one tube to yield a 10% total hydrogen peroxide concentration as a positive control for DNA damage. DMSO was added to one tube for a total of 5% DMSO concentration as a solvent control. Fifty microliters of 840 μ M EE₂ in DMSO was added to another sample to give a final concentration of 32 μ M EE₂. Fifty microliters of 12.5 mM EE₂ in DMSO was added to the 500 μ M tube to give a final EE₂ concentration of 500 μ M. The samples were incubated at room temperature for 15 minutes to allow the treatments to take effect.

Treatment Name	Treatment
None (No Treatment)	Sperm was incubated at room temperature without any additional substances (negative control)
DMSO	DMSO only (solvent control)
32 μ M	32 μ M EE ₂ diluted in DMSO
500 μ M	500 μ M EE ₂ diluted in DMSO
H ₂ O ₂	Hydrogen Peroxide (positive control)

Table 1: Names of each treatment group with descriptions of the treatment.

Comet Assay

In preparation, slides pre-treated to promote adherence of low melting point agarose from the Trevigen CometAssay Kit (Trevigen, Gaithersburg, MD) were warmed in a 37°C incubator to enhance even spreading of samples in agarose. The Lysis Solution (Trevigen CometAssay Kit, proprietary formulation) was chilled at 4°C for at least 20 min before use. Additionally, 250 μ L aliquots of the low-melting agarose Comet LMAgarose (Trevigen CometAssay Kit, proprietary formulation) were melted at 100°C in open Eppendorf tubes for 5 minutes and kept at 37°C in a water bath for at least 20 min to cool. SYBR Green I concentrate in DMSO (Trevigen, Gaithersburg, MD) was diluted 1:10,000 in TE buffer (10mM Tris-HCl, 1mM EDTA; pH 7.5) to make SYBR Green I staining solution and stored at 4°C in the dark.

After preparation, 25 μL of each diluted sperm sample was mixed gently with 250 μL low-melting agarose. Seventy-five microliters of the sperm-agarose mixture was placed onto a comet slide and spread with the flat side of pipette tip, with two samples per slide. The slides were laid flat in the dark at 4°C for at least 10 minutes to solidify. Keeping the temperature at 4°C, the cells were then lysed in the gel by immersing the slide in pre-chilled Lysis Solution for 1 hour. The slides were equilibrated in neutral TBE electrophoresis buffer (10.8 g Tris, 5.5 g boric acid, 4 mL .5M EDTA in 1 L dH₂O; pH 8.0) for 20 minutes. The slides were transferred to horizontal electrophoresis apparatus and positioned along the midline of the tray so that all the samples were equidistant from the electrodes. The electrophoresis machine was run at 1V/cm (15 V for 15-cm tank) for 10 minutes.

After the electrophoresis, the excess buffer was gently tapped off, and the slides were dipped in 70% ethanol for 5 minutes and air-dried. Slides were stored at room temperature in the dark for up to one week until microscope analysis. Fifty microliters diluted SYBR® Green I staining solution was placed onto each sample (circle of dried agarose) on each slide and placed in the dark at 4°C for 5 minutes. Excess stain was tapped off and the slides were dried completely at room temperature in the dark. The slides were viewed under an epifluorescence microscope using the fluorescence filter (521 nm maximum emission) and at least five different fields of vision were photographed with as many cells as possible in focus to obtain at least 100 in-focus cell pictures per sample. These pictures were then manually measured using Zeiss AxioVision software by four different individuals who were uniformly trained. Tail lengths were

compiled into a Microsoft Excel document. At least 100 cells were scored per sample. Across all fish and treatment levels, 4037 comet tails were measured.

Each sperm cell that was photographed and measured was given a unique identification number and labeled by the fish of origin (numbered 1 through 6) and the treatment it received, as well as the day the experiment was run, its location within the electrophoresis chamber, the day on which the tail length was measured, and the person conducting the measurement. Because the size of the electrophoresis chamber only allowed for four treatments to be run for each fish, fishes 1, 2 and 3 received the none, DMSO, 32 μ M, and 500 μ M treatments; fishes 4, 5, and 6 received the DMSO, 32 μ M, 500 μ M, and H₂O₂ treatments

Cell Viability Protocol

The LIVE/DEAD® Sperm Viability Kit (L-7011) from Invitrogen Molecular Probes (Invitrogen, Carlsbad, CA) was used. One-milliliter samples of treated sperm, approximately 1×10^6 cells/mL, were aliquotted into clean 1.5 mL Eppendorf tubes. SYBR-14 stock solution at 1mM SYBR-14 in DMSO was diluted 1:50 in HBSS immediately before use. Five microliters of diluted SYBR-14 was added to each sample for a total concentration of 100 nM and incubated for 10 minutes at room temperature. Five microliters of propidium iodide at 2.4 mM in dH₂O for a final concentration of 12 μ M was added and the samples were incubated an additional 10 minutes at room temperature. The samples were run on the BD FACSAria machine (BD Biosciences, San Jose, CA) at the Maryland Pathogen Research Institute to count cells stained with SYBR-

14 and PI. The blue laser used was provided by the Coherent® Sapphire™ solid state system with an excitation of 488 nm. The machine was calibrated against one unstained, untreated sperm sample; 1 mL of sperm incubated with 8 µL SYBR for 5 minutes at room temperature; and 1 mL of sperm incubated with propidium iodide for 5 minutes at room temperature. Data were recorded using FACSDiva Version 6.1.2 software. Adjustment of gates was also conducted using FACSDiva.

Chapter 4: Results

Statistical Analysis

Since all of the data do not compile into a symmetric matrix, a generalized linear model (GLM) was used to analyze all the data (see Appendix A). The GLM is a statistical model that is a flexible generalization of ordinary least squares regression. The computations were executed using Statistical Analysis Software (SAS) by SAS Institute Incorporated. Two models were run for each of the assays. The first model for the comet assay tested the fish and treatment interaction effects, and the second model estimated the effects of only the treatments. For the Syber-14 assay, the model examined the survival rate for each treatment. The complete set of statistical tests can be found in Appendix A. Note that a relationship is considered significant if its p-value is less than 0.05.

H₀: Short-term exposure to EE₂ is not toxic to hybrid striped bass sperm

H₁: Short-term exposure to EE₂ is cytotoxic to hybrid striped bass sperm

H₂: Short-term exposure to EE₂ produces DNA damage in hybrid striped bass sperm

Each fish must be treated as its own experimental block when examining the statistical results of the comet assay. A consistent set of treatments were not carried on each fish due to limitations from the size of the electrophoresis machine, so fishes 1, 2, and 3 received treatments None, DMSO, 32 μ M, and 500 μ M; while fishes 4, 5, and 6 had treatments DMSO, 32 μ M, 500 μ M, and H₂O₂. The same restrictions on treatments per fish do not apply to the cell viability assay. On April 17, 2009, fish 4, 5, and 6 were

exposed to DMSO, 32 μM , 500 μM , and H_2O_2 and on April 21, 2009, fish 7, 8, and 9 were exposed to all treatments. Analyses of the results are based off of the treatments and not dependent on the fish number.

Genotoxicity Assay

The first model was a Sum of Squares Type III Test (Appendix A) to determine whether the interaction between the fish and the treatment was significant. This interaction was found to be significant; therefore, the data was not pooled by treatment group because the fish from which each sperm sample was collected significantly impacted the comet tail length for each treatment group. This treatment interaction effect was predicted by our experimental design, wherein cells collected from a single fish were run together in an electrophoresis chamber, separately from the other fishes' samples, ensuring that the exact conditions of the assay were maintained for all samples taken from a single fish but not between different fish. Because each fish's samples were run in a separate electrophoresis chamber, it is not valid to compare data collected between fish, as minute differences in each electrophoresis run (mainly duration and positioning of the slides) have a large effect on the final electrophoresis tail lengths. The decision not to compare data between fish and to view the six fish as replicates of the same experiment was therefore validated by the significant fish-treatment interaction found through the Sum of Squares Type III Test (Appendix A). The average tail length in micrometers for each fish and treatment group are as follows, in tabular and graphical format, as determined by the Type III Test of Fixed Effects (Appendix A):

Fish	Treatment	Mean (μm)	Standard Deviation (μm)
1	None	71.44122	14.91945
1	DMSO	109.8011	16.85261
1	32 μM	108.5602	12.52097
1	500 μM	91.8214	20.30731
2	None	57.36966	12.29593
2	DMSO	56.02131	13.14425
2	32 μM	55.71535	14.71346
2	500	64.77337	15.69768
3	None	56.81151	12.02249
3	DMSO	76.27872	15.77025
3	32 μM	57.17697	11.81094
3	500 μM	49.79749	11.40173
4	DMSO	92.90819	11.80768
4	32 μM	102.5059	12.21079
4	500 μM	96.06584	13.7418
4	H ₂ O ₂	99.01569	13.68102
5	DMSO	100.9357	15.84835
5	32 μM	90.28136	13.45648
5	500 μM	85.00576	13.98685
5	H ₂ O ₂	82.54812	13.82062
6	DMSO	126.112	13.60589
6	32 μM	97.80489	13.29715
6	500 μM	97.53685	14.24382
6	H ₂ O ₂	130.4072	18.02533

Table 2: Average genetic damage for each fish and treatment group.

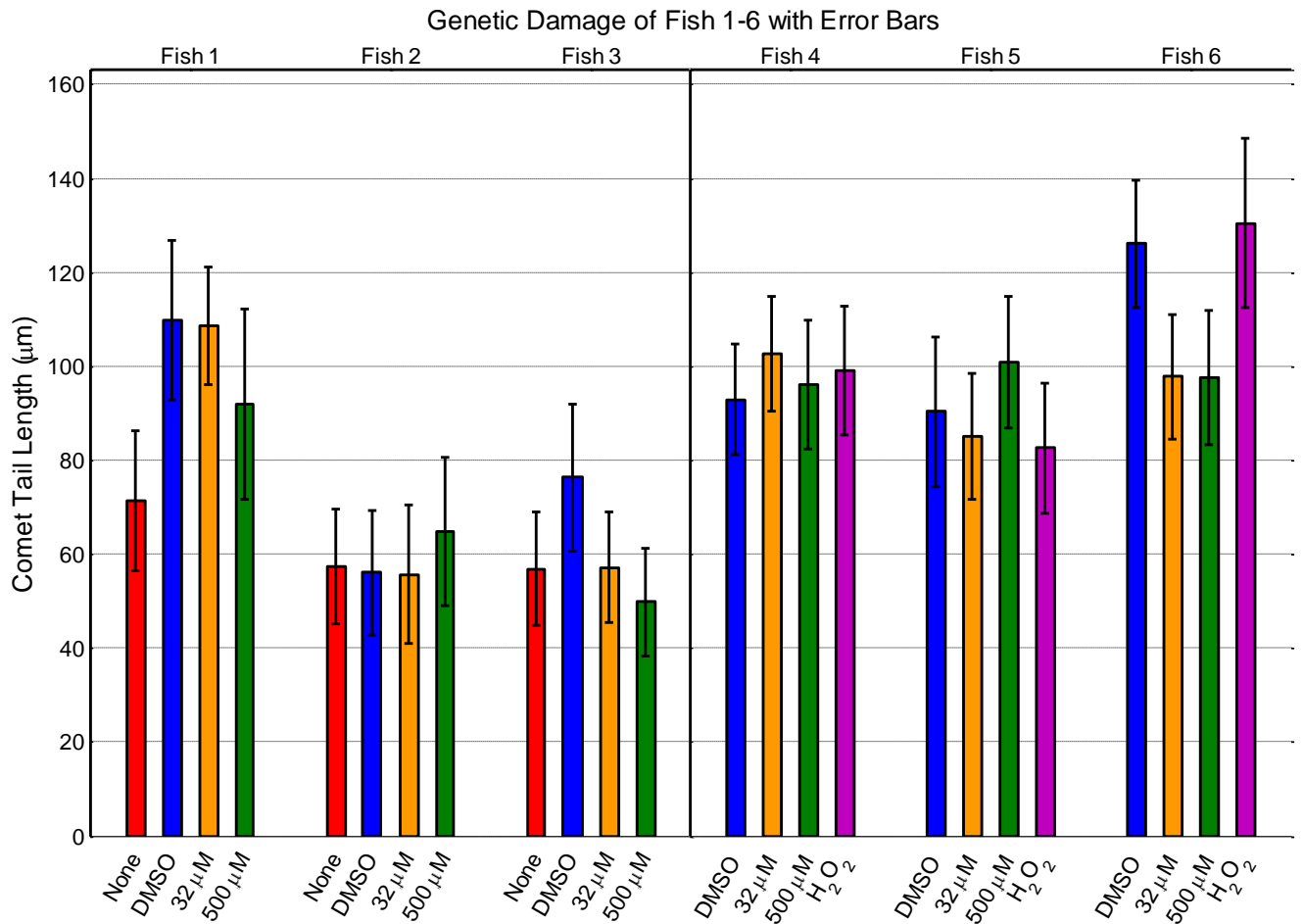


Figure 3: Comet assay results for average comet tail length, an indicator of genetic damage, grouped by fish and treatment group. Longer tail lengths indicated more double-strand DNA breaks and therefore more genetic damage. Fish were treated as separate experimental blocks, and results between fish should not be compared.

The standard deviation is relatively high for each treatment group, demonstrating a high degree of variability in the data. The average tail length for cells within a treatment group was compared to the average tail length of cells in the DMSO and none treatment groups for that fish using a series of pairwise t-tests. While differences between tail lengths in certain treatment groups were found to be significant in particular fish, the results were not replicable in other fish. For instance, the average tail lengths of

cells in the 500 μM treatment group in fishes 1, 3, 5, and 6 were significantly shorter than those of the DMSO-only treatment groups. Due to the lack of consistency, the data are not able to support a significant difference in genotoxicity, as measured by comet tail length, in cells exposed to various amounts of EE_2 .

Treatment 1	Treatment 2	Difference in Averages (μm)	p-value
DMSO	32 μM	-0.48271	0.6293
DMSO	500 μM	-6.12986	<.0001
DMSO	None	-14.5485	<.0001
32 μM	None	-19.0823	<.0001
500 μM	None	-8.47781	<.0001

Table 3: Pairwise T-Tests to control for Fish 1. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

Treatment 1	Treatment 2	Difference in Averages (μm)	p-value
DMSO	32 μM	-0.27685	0.7819
DMSO	500 μM	7.195564	<.0001
DMSO	None	1.239563	0.2152
32 μM	None	1.305533	0.1918
500 μM	None	-5.42325	<.0001

Table 4: Pairwise T-Tests to controls for Fish 2. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

Treatment 1	Treatment 2	Difference in Averages (μm)	p-value
DMSO	32 μM	-14.3349	<.0001
DMSO	500 μM	-18.4841	<.0001
DMSO	None	-13.6051	<.0001
32 μM	None	-0.28338	0.7769
500 μM	None	5.035803	<.0001

Table 5: Pairwise T-Tests to controls for Fish 3. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

Treatment 1	Treatment 2	Difference in Averages	p-value
DMSO	32 μ M	7.746974	<.0001
DMSO	500 μ M	2.345486	0.0191
DMSO	H ₂ O ₂	4.648509	<.0001

Table 6: Pairwise T-Tests to controls for Fish 4. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

Treatment 1	Treatment 2	Difference in Averages	p-value
DMSO	32 μ M	-6.71098	<.0001
DMSO	500 μ M	-8.60861	<.0001
DMSO	H ₂ O ₂	-10.7155	<.0001

Table 7: Pairwise T-Tests to controls for Fish 5. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

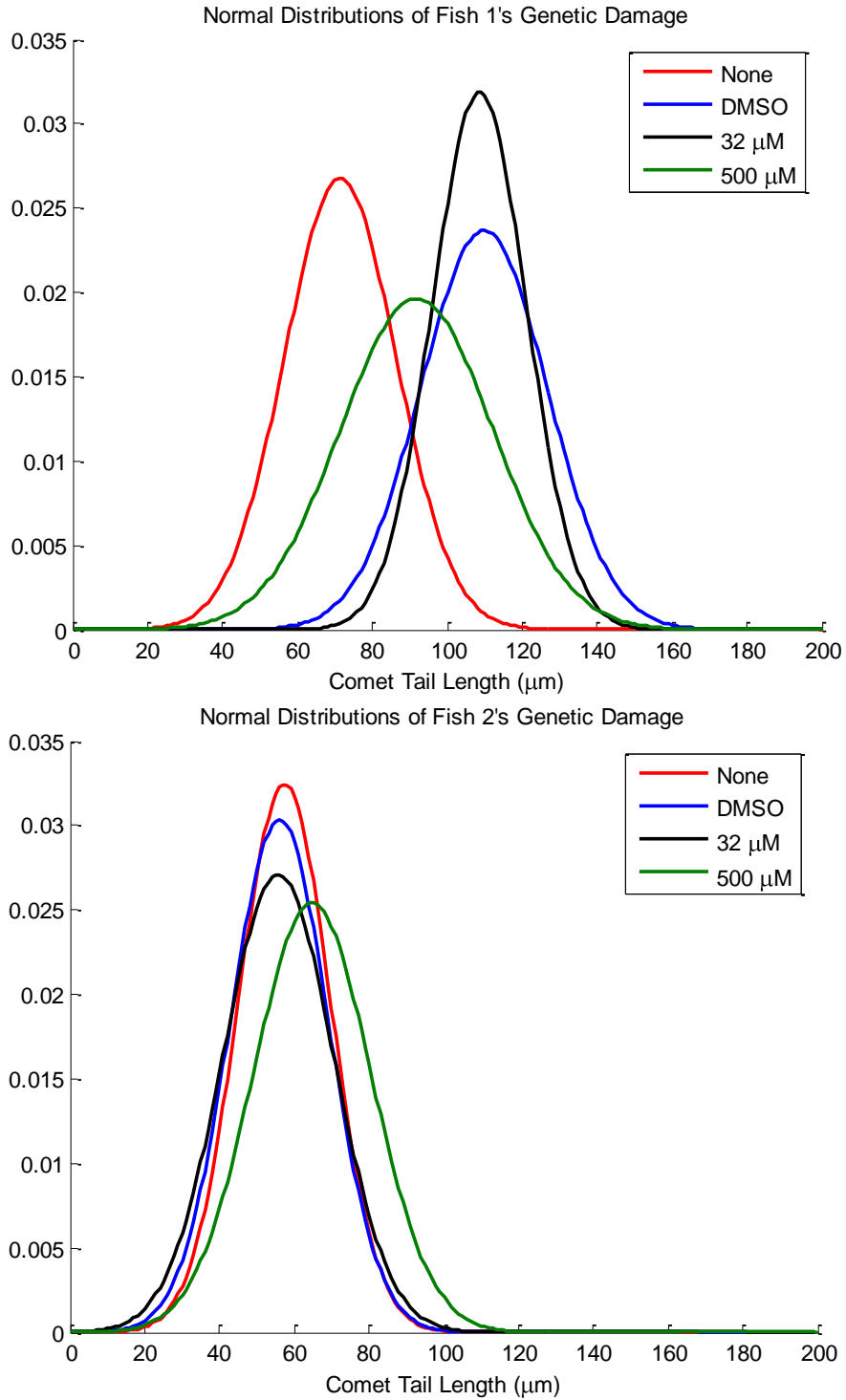
Treatment 1	Treatment 2	Difference in Averages	p-value
DMSO	32 μ M	-14.8972	<.0001
DMSO	500 μ M	-15.1331	<.0001
DMSO	H ₂ O ₂	2.052256	0.0402

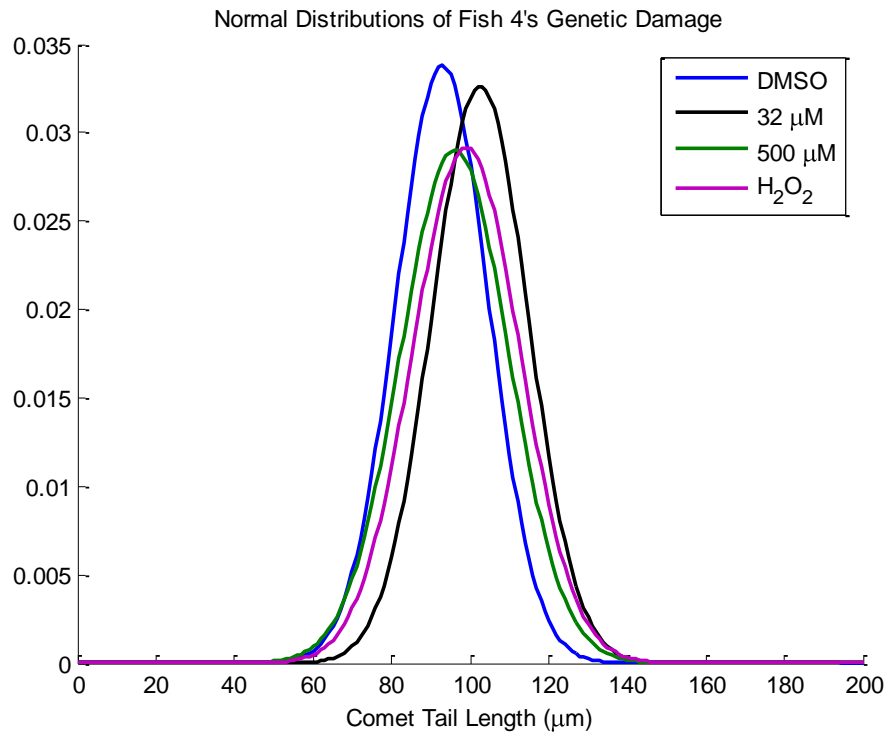
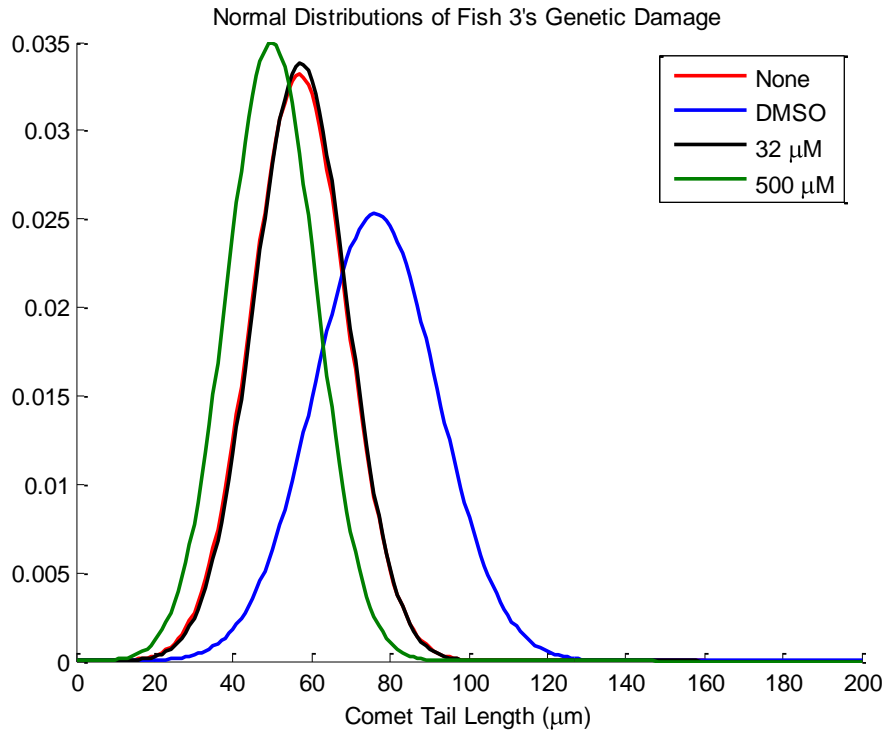
Table 8: Pairwise T-Tests to controls for Fish 6. The difference in averages is equal to the average tail length of cells in treatment two minus the average tail length of cells in treatment one.

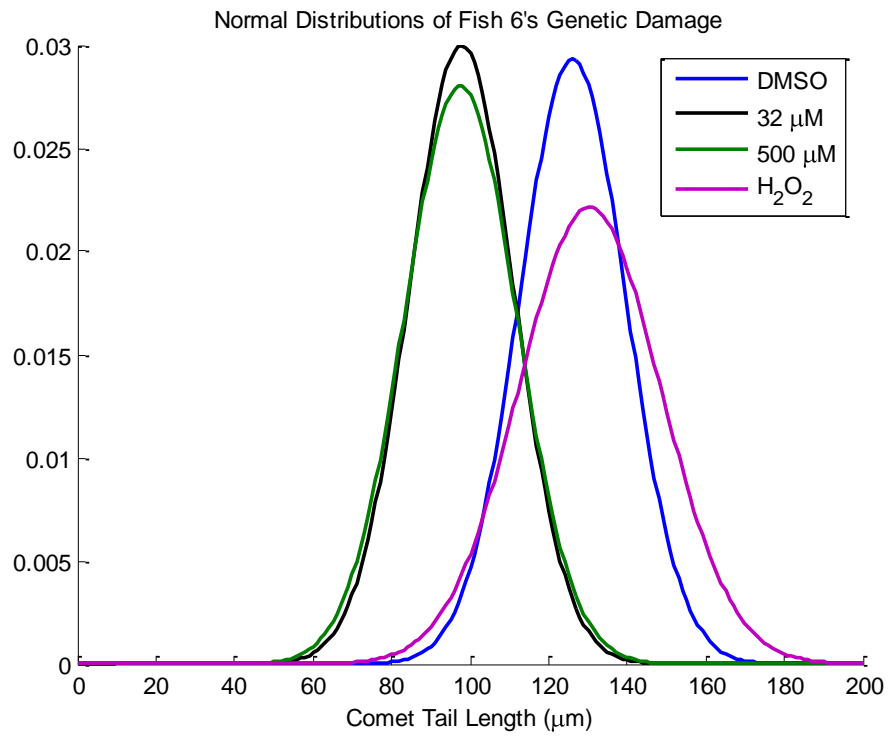
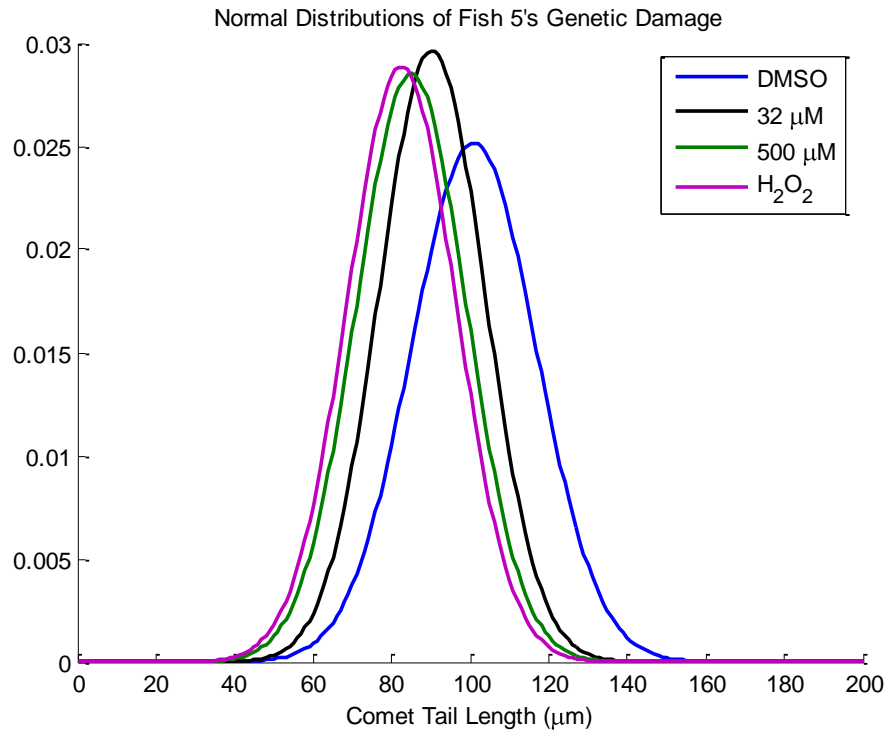
The following are a series of Gaussian distributions for each fish. The dataset for each fish-treatment group is represented as a normal curve, where the maximum is located at the mean tail length and the spread of the curve is directly dependent upon the standard deviation. Two perfectly overlapping curves are identical, and the less area shared beneath two curves, the more likely the datasets are to be significantly different.

If all treatments produce cells with significantly different tail lengths, the curves for each treatment group would not overlap.

Figure 4: Gaussian Distributions of Average Genetic Damage for Fish







Viability Assay

After treatment with EE_2 and staining with SYBR-14 and PI, samples were subjected to FACS analysis in order to measure the fluorescence of thousands of cells. Below is a sample of the data, the full set can be found in Appendix C.

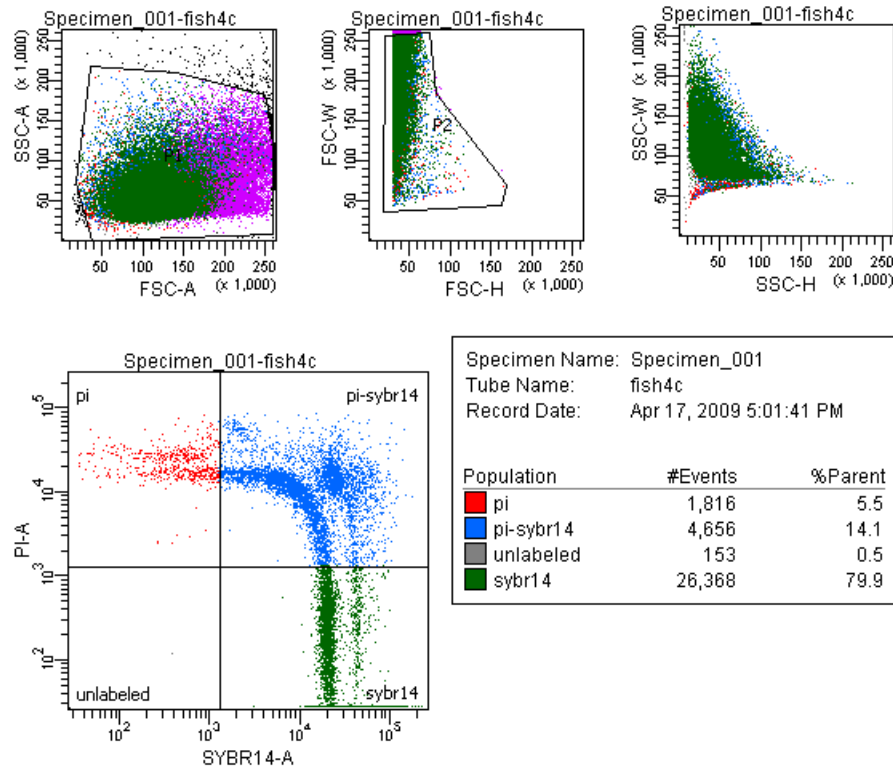


Figure 5: Sample of FACS data from fish #4, treatment group 32 μ M EE_2 .

As expected, the sample seemed to be comprised of mostly one homogenous population of cells based on the forward and side scatter values, and so the gating included most of the events counted by the FACS system. Tens of thousands of cells were counted in each sample. A green fluorescence due to SYBR-14 staining (quadrant IV) signified intact cell membranes and thus that the cell was alive. Red fluorescence

due to PI staining (quadrant II) signified ruptured cell membranes and thus cell death. Cells that fluoresced both green and red (quadrant I) were considered moribund as PI can only enter cells with a ruptured membrane; hence, the totals for all cells stained with PI were summed together and considered dead when calculating survival rate. Cells that emitted green fluorescence only were considered alive. Cells that emitted no fluorescence (quadrant III) were considered to be pieces of cell-sized debris, and this data was omitted entirely from statistical analysis.

In the case of the 500 μM EE₂ treatment, the FACS machine detected very low levels of fluorescence in all cells in fishes 7, 8, and 9. Gating was recalibrated for these fish/treatment groups to reflect the overall levels of fluorescence before counting the number of cells that were alive or dead. See Appendix C for original and recalibrated data.

Survival rate was calculated as the percentage of living cells out of the total number of cells for each treatment group.

$$\text{Survival Rate} = \frac{\# \text{ live}}{\# \text{ live} + \# \text{ dead}}$$

An average survival rate was calculated for each treatment by pooling the data from all six fish.

Treatment	Mean Survival Rate	Standard Error
H ₂ O ₂	0.3130	.09041
500 μM	0.4828	.09041
DMSO	0.7753	.09041
32 μM	0.7960	.09041
None	0.8570	.1279

Table 9: Mean Survival Rates for Each Treatment

The survival rates for each treatment were compared using pairwise T-tests, shown in Table 5, after running a Type III Test of Fixed Effects model (Appendix A). The positive control for cytotoxicity, treatment with H₂O₂, was found to have significantly decreased survival rate in comparison to the “none” control (p = 0.0029). This confirms that our assay was sufficiently sensitive to detect cytotoxic damage. The “none” and DMSO treatments were found to not be significantly different from each other (p = 0.6083), indicating the solvent did not have a significant effect on cell viability by itself. The survival rate of cells treated with 500 μM EE₂ was significantly lower than cells treated with the DMSO control (p = 0.0353), indicating a cytotoxic effect at this very high level of EE₂. The 32 μM treatment did not cause significantly more damage than the DMSO control (p = 0.8730).

Treatment	Treatment	Difference in Survival Rate	p-value
32 μ M	DMSO	0.02075	0.8730
32 μ M	None	-0.06101	0.7017
500 μ M	DMSO	-0.2924	0.0353*
500 μ M	None	-0.3742	0.0287*
DMSO	None	-0.08176	0.6083
H ₂ O ₂	None	-0.5440	0.0029*

Table 10: Pairwise comparisons of cell survival rates as measured by differential live/dead staining and FACS analysis.

Chapter 5: Discussion

Comet Assay Conclusion

When the data from the comet assay were analyzed using treatment as the only independent variable, the data seemed to suggest that EE₂ had a genotoxic effect on sperm cells. When the fish that the samples came from was also treated as an independent variable, it became clear that within each fish there was so much variation that the positive and negative controls did not always produce the most and the least damage respectively. The Gaussian distributions of each fish's genetic damage (see Figure 2 in the results section) demonstrate that the positive and negative controls did not elicit the genetic damage that was hypothesized. For example, the H₂O₂ treatment, which was the positive control, caused the lowest measured DNA damage in sperm collected from fish 5. In the sperm collected from fish 3, the none control, which should have caused the lowest genetic damage, was measured to have the second highest amount of damage among all of the treatments. Without the positive and negative controls measuring to have caused the highest and lowest DNA damage to sperm cells, no conclusions can be drawn from the comet assay.

The electrophoresis chamber had only eight wells, so in order to run samples in duplicate, each sample could only be exposed to four treatments. In order to use five treatments (none, DMSO, 32 μM, 500 μM, and H₂O₂), three fish were run without a positive control and three fish were run without a none treatment. The difficulties

inherent in manually determining comet tails were a source of variability in the comet tail length measurements. There were many poorly focused photographs which meant that the unfocused photographs appeared to have shorter tails than slides that were in-focus. These slides were still unfocused even after using the Zeiss Axiovision software to improve the quality of the image. Fish 2 and 3 had very unfocused pictures and the tail lengths were measured to be much shorter than the comet tails from any other fish. Fish 2 and 3 did not have a positive control treatment, so the data from fish 2 and 3 significantly reduced the least-squared-mean of the none treatment. The only other data for this treatment was from fish 1, so approximately two-thirds of the data for the none control were measured on unfocused images, and so many of the comet tail lengths for the none treatment were smaller than their actual tail lengths.

The positive H₂O₂ control was a treatment only for fish 4, 5, and 6—all of which had slides that were relatively in focus. This makes the lengths of the comet tails from this treatment comparatively longer on average because it was the only treatment that had all focused slides. The three other treatments: DMSO, 32 μM, and 500 μM were given to all six fish, so approximately two-sixths of the slides from these treatments were unfocused. Therefore, the data had a lot of variability with the positive control having the most accurately represented tail length measurements. The other treatments had smaller measurement values than their actual tail lengths with the none treatment values being the most deflated. This gave the misleading appearance that the data matched the hypothesis when the source fish was ignored and treatment was considered the only independent variable.

The comet assay was complicated by several logistical limitations. Because EE₂ is lipophilic, it needed to be mixed with a solvent in order to directly expose the sperm. DMSO was chosen because of its wide use in sperm cryopreservation and because numerous studies have indicated that it is not damaging to sperm (Taitson, 2008; Vivieros 2009). In addition, a 2000 study used DMSO as a solvent to examine the DNA of a bushcricket (Simmons, 2000). This study indicated that DMSO was not damaging to DNA. Nonetheless, to determine whether the use of DMSO had any effect on our results we needed to use two separate controls. The carrier control had DMSO but no EE₂ to isolate the effects of the EE₂ treatment. Another control, labeled as the “none” treatment, had no DMSO to determine if the DMSO solvent affected our results. For these reasons we had five treatment levels: two negative controls, a positive control, and two EE₂ treatments. Therefore, it was not feasible to run all treatment groups in the same eight-well electrophoresis chamber.

Another logistical limitation to our experiment was that the hybrid striped bass were only spermiating for a limited period of time. Because all of the samples needed to be collected within that time window, the number of possible replications was limited.

The practical difficulties experienced in performing the comet assay on our samples and interpreting the results may explain the high level of variability in our data. Practically, EE₂ could not be added to every sperm sample at the same exact time, so the first samples to have the treatment solution added were incubated for a slightly longer amount of time. This time difference could have had an important effect on the DNA damage we measured because the bass sperm cells begin to degrade as soon as the semen is collected. Another logistical problem that may have been a source of error was having

to stain and photograph the comet slides on different days. According to Trevigen's instructions, the comet slides can be viewed up to two weeks after electrophoresis has been run. However, it is possible that there were differences in the comet tail lengths on different slides as a result of the different amounts of time that passed before photographs were taken. There may also have been an effect from the amount of time we spent taking photographs after the fluorescent dye had been added to the slides. The fluorescence started to photobleach as soon as the dye was added, and the brightness of the fluorescence affected the apparent length of comet tails. Due to these numerous problems, the results of the comet assay were highly variable and inconclusive.

SYBR-14 Assay Conclusion

Our study shows that at high concentrations, short-term exposure to EE₂ can cause cytotoxic damage to sperm cells. At an alpha level of 0.05, the 500 μM treatment was found to cause significantly more cytotoxic damage than the none treatment or carrier control, but the 32 μM treatment did not cause significantly more damage than the controls. This suggests that at some concentrations EE₂ is not cytotoxic but at very high concentrations it is.

Flow cytometry is a very powerful tool which not only enabled the measuring of thousands of cells per sample, but also avoids the subjectivity of researchers counting red and green cells by hand. This allows for much more reliability in results than in the comet assay. Different treatments were used on the two different days that this assay was run. The first day, there was no "none" treatment but on the second day all five were run.

Over tens of thousands of data points were measured for the viability assay. For our study, this is probably one of the reasons why the results are more consistent.

The flow cytometry machine needed to be recalibrated for the 500 μM treatment. When run with the same parameters for particle size and fluorescence level as the other treatments, the flow cytometer did not detect any fluorescence from over 99.4% of the cells from fish 7, 8 and 9 exposed to the 500 μM EE₂ treatment. Although the reason for this is unknown, it is possible that the high concentration of estrogen affected the fluorescence of the stains. The recalibrated data was used in statistical analysis.

Environmental Relevance

The concentrations of EE₂ used in this study are higher than levels found in natural waterways. Natural levels are normally found in the nanograms per liter range (Shved et al., 2008) whereas this study used levels that were many orders of magnitude higher. Studies have shown that environmental levels of EE₂ can decrease fertility and fecundity (Nash et al., 2004) in fish and can lead to population collapse (Kidd et al., 2007). Both of these studies exposed fish over multiple generations. Kidd et al. found that in a lake environment, multigenerational exposure of 2 ng/L of EE₂ to a fish population resulted in population collapse after three generations.

There are a number of possible causes for this population collapse including disrupted sexual development, disrupted development of gametes, embryo death, and lowered gamete viability. First, as an EDC, EE₂ has a potential of disrupting the

hormonal balance of the HPG axis that affects the sexual development in fish. In the Nash et al. study, several males from the F₁ generation did not fully develop sexually. Some males had no functional testes after a lifetime exposure to EE₂ at 5 ng/L. Furthermore, EE₂ concentrations as low as 1 ng/L have been demonstrated to reduce the male to female ratio within a given population of Chinese rare minnows (Zha et al., 2008). Second, EE₂ has also been correlated with reduced number of spermatozoa in zebrafish (Xu et al., 2008). Third, EE₂ has also been implicated in embryo death. In one study, 100% of embryos exposed to 7 ng/L of EE₂ for 60-70 hours post-spawning died (Gabi Dumitrescu et al, 2009). Lastly, direct exposure of EE₂ on sperm or eggs may have an effect on gamete viability.

The data from this experiment suggests that direct short-term exposure of EE₂ at 500 µM can cause cytotoxic damage in sperm cells, whereas direct exposure of EE₂ at 32 µM probably does not cause cytotoxic damage in sperm cells. 32 µM is several orders of magnitude higher than the concentrations used in these previous studies that demonstrate population collapse. This indicates that the cytotoxic effect of EE₂ on sperm cells does not contribute to reduced fertility of affected fish populations. Therefore, the pathway by which EE₂ affects reproductive efficacy is not likely to be through direct exposure to sperm cells, but rather through a different mechanism. There are many other endpoints of reproductive efficacy, including sperm DNA integrity that this experiment attempted to address. Unfortunately, the comet assay data was inconclusive, and more experiments will be required to further elucidate the exact effects of EE₂ on male fish reproductive efficacy and the mechanisms in which the fish populations collapsed when exposed to EE₂ (Nash et al., 2004; Kidd et al., 2007).

The negative effects that EE₂ exposure can cause to fish populations can also be induced in fish exposed to sewage effluent. In an experiment performed by Filby, et al., fathead minnows were exposed to three types of treatments: sewage effluent only, effluent and EE₂, and EE₂ only (Filby et al., 2007). The effluent exposure inflicted genotoxic damage when exposed to both blood and gonadal cells. However, only the males exhibited a genotoxic damage in gonadal tissue when exposed to EE₂ alone. This experiment shows not only the negative effect of sewage effluent, but also the increased effect of EE₂ on the cells, which is important considering that sewage effluent is a considerable source of EE₂ and other synthetic estrogens (Kuster et al., 2004).

Downstream of Wastewater Treatment Works (WWTW) in London for the past quarter century, intersex roach have been appearing in greater numbers (Tyler & Jobling, 2008). In Tyler and Jobling's field study, a positive correlation was drawn between concentration of effluent in water and percentage of wild intersex roach. Specific observations include that roach located downstream of multiple WWTW sites were between 16% and 100% intersex, compared to between 11% and 44% of roaches located from upstream sites (Jobling et al., 1998). At the sites themselves, scientists recorded an intersex rate of 86% (Jobling et al., 2006). This experiment ties together with Filby's experiment.

Endocrine disruptors are not only affecting the reproductive efficacy of fish. Rats exposed to endocrine disruptors in their drinking water experience lower birthrates than unexposed rats (Vosges et al., 2008). Other studies have demonstrated that sperm levels have decreased over time in human males (Murray, 2002). Phthalates have been found in human breast milk which can lead to sexual development problems in children (Lottrup,

2005). Endocrine disruptors clearly affect the reproductive health of fish and mammalian species. Future research is required to determine the pathways by which these chemicals harm gamete viability and other measures of reproductive efficacy.

Suggestions for Future Experiments

The range of future experiments that build on the scientific findings and environmental underpinnings of our experiment are wide. From a micro view, our experiment could be amended in numerous ways to increase the value of the information received. To begin with, future experiments could be designed to more specifically examine the endpoints of cytotoxic and genotoxic damage on sperm cells. As our results were in many ways inconclusive, more reliable tests could be designed to better describe how estrogenic compounds affect hybrid bass sperm cells. A further endpoint of sperm viability is sperm motility. Our experiment focused on whether the sperm cell was alive, dead or genetically damaged, but did not test for how well the sperm cell functions *in situ* as it attempts to fertilize an egg. If a sperm cell cannot properly move, it is not an effective reproducer. Further experiments could examine this trait, possibly by using a Computer Assisted Sperm Analysis (CASA) system to measure how efficiently individual sperm cells move. Genomic integrity, motility, and viability to fertilize an egg are all important measures of sperm health.

The most probable sources of error in this experiment were the inconsistencies in the methodology. Our genotoxicity assay should be reattempted with a modified protocol. For logistical reasons, the experiment we conducted was on two different days

by different people. Different treatment levels were used on different days. Pictures were taken of the comet tail slides over a two-week period and the comet tails were measured by five different people. If this study is reattempted, the methodology should be updated so that all of the samples are collected on the same day. Each sample of sperm should be exposed to the same number of treatments: a none control, a DMSO-only carrier control, each EE₂ treatment, and a positive control. One way to facilitate this is to use a larger electrophoresis chamber with enough wells so that all samples of each treatment can be run in duplicate. To cut down on variability, each task should be performed by the same people each time. The exposure times should be staggered by about 30 minutes to ensure that each sample is incubated for the same amount of time. The comet tails should be measured by only one person to adjust for some of the inherent subjectivity in measuring. With these modifications, the comet assay might provide more conclusive results that indicate a link between short-term exposure to EE₂ and DNA damage in sperm.

To further understand the mechanisms by which EDCs can limit fish populations, reproductive health needs to be studied in longer-term experiments similar to the one mentioned in the appendix (Appendix E) of this thesis or multigenerational experiments similar to the one conducted by Nash et al. (2004). Sperm health, as measured by cytotoxicity, genotoxicity, and motility should be compared between fish that have been exposed to various concentrations of EE₂. These experiments are more environmentally relevant and could indicate which of the three endpoints might contribute to population decline. The appendix outlines a preliminary experiment in which male fathead minnows were exposed by injection to various concentrations of EE₂. The fish used in the study

were unfortunately infected with blood flukes and many died prematurely before enough data was collected to draw conclusions. If this study was reattempted with a healthier population, then it could help elucidate the mechanisms by which estrogens reduce fish populations.

Even though this study shows that short-term exposure to high concentrations of EE₂ can cause cytotoxic damage to sperm cells, this relationship should be studied using sperm cells and other types of cells with several low and high concentrations of EE₂. Our flow cytometry data counted almost no fluorescence for cells exposed to the highest concentration of EE₂. To determine the effect of EE₂ on stain fluorescence, healthy cells should be exposed to solutions of dyes mixed with EE₂. Unlike our study, the cells should not incubate in EE₂ any longer than is needed for the stains to work. According to the findings of this study, the EE₂ should not have statistically significant effects on the cells, and so the flow cytometry data should measure only the effects that the EE₂ has on the dyes. A recent study of sperm cryopreservation found that methanol and DMSO have different effects on the fluorescence of stains (Psenicka et al., 2008). As a follow-up to the Psenicka study, both methanol and DMSO should be used as solvents. The results of such a study could inform the methodology of future experiments using stains with estrogens and DMSO.

Other approaches include altering the experimental design in broader ways. Different techniques could be used to make the experiment more environmentally relevant with, for instance, *in vivo* studies. Instead of exposing sperm themselves, the fish could be exposed to estrogenic compounds. This could be done by injecting fish with different levels or types of estrogens, or creating a more representative environmentally-

relevant flow-through system. A flow through system is a self-contained aquatic environment. Water would be constantly moved throughout a system of fish tanks, and controls could be made for precise water quality. This means that different treatment groups could be exposed to different levels of estrogenic compounds in the water they inhabit. Aside from studying fish in their natural environment or using man-made lakes, this would be the closest approximation of a fish's natural habitat.

From the macro view, there is no limit on the studies that can be conducted. The end-goal of our study was to contribute to the literature in some form, especially to add understanding to how estrogenic compounds released into the wild (usually through man) affects fish, other animals and the ecosystems in which they live. Further experiments can tackle all of these issues. Studies can examine other fish species as well as mammalian, amphibian and reptilian species that may be in environments similarly affected by estrogens. Experiments could examine their sperm, as we did, or other biological or even behavioral affects that could be produced. It is simply not known how harmful estrogenic compounds can be to animal species, and any experimental design to add to this discussion is helpful.

It has been shown that waste-water treatment centers, while they remove most harmful elements from treated water, are not able to remove estrogenic compounds. The additive effect of these compounds in nature complicates the overall problem. If this has been shown to affect the environment and animals, how might it affect humans? After all, this is the same water used to irrigate farms, which feed and support the animals that humans then later consume. It is also the same water people drink, bathe in and cook with. Can these compounds affect humans? This is especially alarming if indeed

exposure to estrogenic compounds decreases the reproductive viability of those organisms it affects. Again, the variety of experiments that can and should be conducted to shed light on these questions are effectively unlimited. Experiments can look at ways to decrease or eliminate estrogens from passing through waste-water treatment plants. Most importantly, studies can be designed to examine how humans might be affected. Tests could be made to see just how much estrogen is in our drinking water, or the total amount of estrogen we ingest through the variety of sources mentioned above. Experiments should also be carried out to determine to what degree we are biologically affected by these compounds, and should not be limited to reproductive viability, although that should be a centerpiece. In the end, anything that contributes to the scientific literature surrounding these broad questions would be helpful and add to our understanding of this burgeoning problem.

Appendix A: SAS Output

Part I: Comet

The 1st model was to test for Fish x Treatment interaction effects.

Comet data model I

Class Level Information

Class	Levels	Values
Fish	6	1 2 3 4 5 6
treatment	5	32uM 500uM DMSO H2O2 none

Number of Observations Read	4037
Number of Observations Used	4037

Dependent Variable (y): Comet

Sum of Source	DF	Squares	Mean Square	F Value	Pr > F
Model	23	1933886.842	84082.037	452.66	<.0001
Error	4013	745421.641	185.752		
Corrected Total	4036	2679308.482			

R-Square	Coeff Var	Root MSE	Comet Mean
0.721786	17.28405	13.62908	78.85351

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	4	424767.673	106191.918	571.69	<.0001
Fish*treatment	19	1549540.288	81554.752	439.05	<.0001

Comet Least Squares Means

treatment	LSMEAN	
	Comet LSMEAN	Number
32uM	85.340784	1
500uM	80.833451	2
DMSO	93.676153	3
H2O2	103.990334	4
none	61.874132	5

Fish	treatment	LSMEAN	
		Comet LSMEAN	Number
1	32uM	108.560185	1
1	500uM	91.821400	2
1	DMSO	109.801053	3
1	none	71.441222	4
2	32uM	55.715354	5
2	500uM	64.773372	6
2	DMSO	56.021312	7
2	none	57.369662	8
3	32uM	57.176966	9
3	500uM	49.797487	10
3	DMSO	76.278721	11
3	none	56.811510	12
4	32uM	102.505943	13
4	500uM	96.065839	14
4	DMSO	92.908191	15
4	H2O2	99.015690	16
5	32uM	90.281361	17
5	500uM	85.005758	18
5	DMSO	100.935667	19
5	H2O2	82.548120	20
6	32uM	97.804894	21
6	500uM	97.536849	22
6	DMSO	126.111975	23
6	H2O2	130.407191	24

Part I: Comet (cont'd)

The 2nd model estimates the real effects from treatments.

Comet data model II

Class Level Information

Class	Levels	Values
Fish	6	1 2 3 4 5 6
treatment	5	32uM 500uM DMSO H2O2 none

Number of Observations

Number of Observations Read	4037
Number of Observations Used	4037
Number of Observations Not Used	0

Type 3 Tests of Fixed Effects

Effect	Num Den		F Value	Pr > F
	DF	DF		
treatment	4	19	168.81	<.0001

Least Squares Means

Effect	treatment	Comet		DF	t Value	Pr > t
		Estimate	Standard Error			
treatment	32uM	80.6532	0.7051	19	114.39	<.0001
treatment	500uM	77.3699	0.8337	19	92.81	<.0001
treatment	DMSO	79.3348	0.7011	19	113.16	<.0001
treatment	H2O2	100.54	1.1989	19	83.86	<.0001
treatment	none	59.6033	1.0472	19	56.92	<.0001

Differences of Least Squares Means

Effect	treatment	treatment	Standard		DF	t Value	Pr > t
			Estimate	Error			
treatment	32uM	500uM	3.2833	1.0918	19	3.01	0.0072
treatment	32uM	DMSO	1.3184	0.9943	19	1.33	0.2006
treatment	32uM	H2O2	-19.8869	1.3908	19	-14.30	<.0001
treatment	32uM	none	21.0499	1.2625	19	16.67	<.0001
treatment	500uM	DMSO	-1.9649	1.0893	19	-1.80	0.0871
treatment	500uM	H2O2	-23.1702	1.4602	19	-15.87	<.0001
treatment	500uM	none	17.7666	1.3385	19	13.27	<.0001
treatment	DMSO	H2O2	-21.2052	1.3888	19	-15.27	<.0001
treatment	DMSO	none	19.7315	1.2602	19	15.66	<.0001
treatment	H2O2	none	40.9367	1.5919	19	25.72	<.0001

Part II: Live/Dead

First model used **Ratio = live:dead** as the dependent variable (y).

Class Level Information

Class	Levels	Values
Fish	6	4 5 6 7 8 9
treatment	5	32uM 500uM DMSO H2O2 none

Number of Observations Read	27
Number of Observations Used	27

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
treatment	4	17	4.18	0.0154

treatment Least Squares Means

treatment	Ratio Estimate	Standard Error	DF	t Value	Pr > t
32uM	4.2868	0.8911	17	4.81	0.0002
500uM	2.6962	0.8911	17	3.03	0.0076
DMSO	4.2043	0.8911	17	4.72	0.0002
H2O2	0.7143	0.8911	17	0.80	0.4339
none	6.2446	1.2602	17	4.96	0.0001

Differences of treatment Least Squares Means

treatment	treatment	Standard Estimate	Error	DF	t Value	Pr > t
32uM	500uM	1.5906	1.2539	17	1.27	0.2217
32uM	DMSO	0.08249	1.2539	17	0.07	0.9483
32uM	H2O2	3.5725	1.2539	17	2.85	0.0111
32uM	none	-1.9578	1.5382	17	-1.27	0.2202
500uM	DMSO	-1.5082	1.2539	17	-1.20	0.2456
500uM	H2O2	1.9819	1.2539	17	1.58	0.1324
500uM	none	-3.5484	1.5382	17	-2.31	0.0339
DMSO	H2O2	3.4901	1.2539	17	2.78	0.0127
DMSO	none	-2.0403	1.5382	17	-1.33	0.2023
H2O2	none	-5.5303	1.5382	17	-3.60	0.0022

Part II: Live/Dead (cont'd)

Second, using **Survival= live/(live+dead)** as the dependent variable (y).

Class Level Information

Class	Levels	Values
Fish	6	4 5 6 7 8 9
treatment	5	32uM 500uM DMSO H2O2 none

Number of Observations Read	27
Number of Observations Used	27

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
treatment	4	17	6.00	0.0034

treatment Least Squares Means

treatment	Survival Estimate	Standard Error	DF	t Value	Pr > t
32uM	0.7960	0.09041	17	8.80	<.0001
500uM	0.4828	0.09041	17	5.34	<.0001
DMSO	0.7753	0.09041	17	8.58	<.0001
H2O2	0.3130	0.09041	17	3.46	0.0030
none	0.8570	0.1279	17	6.70	<.0001

Differences of treatment Least Squares Means

treatment	treatment	Standard Estimate	Error	DF	t Value	Pr > t
32uM	500uM	0.3132	0.1279	17	2.45	0.0254
32uM	DMSO	0.02075	0.1279	17	0.16	0.8730
32uM	H2O2	0.4830	0.1279	17	3.78	0.0015
32uM	none	-0.06101	0.1566	17	-0.39	0.7017
500uM	DMSO	-0.2924	0.1279	17	-2.29	0.0353
500uM	H2O2	0.1698	0.1279	17	1.33	0.2018
500uM	none	-0.3742	0.1566	17	-2.39	0.0287
DMSO	H2O2	0.4622	0.1279	17	3.62	0.0021
DMSO	none	-0.08176	0.1566	17	-0.52	0.6083
H2O2	none	-0.5440	0.1566	17	-3.47	0.0029

Appendix B: Samples of Comet Assay Slides

The photographs of the comet slides varied in quality. Tails were measured only if they were in the plane of focus, and it was easy to see the beginning and end of the comet. Some slides had many tails in the plane of focus while others have no tails in focus.

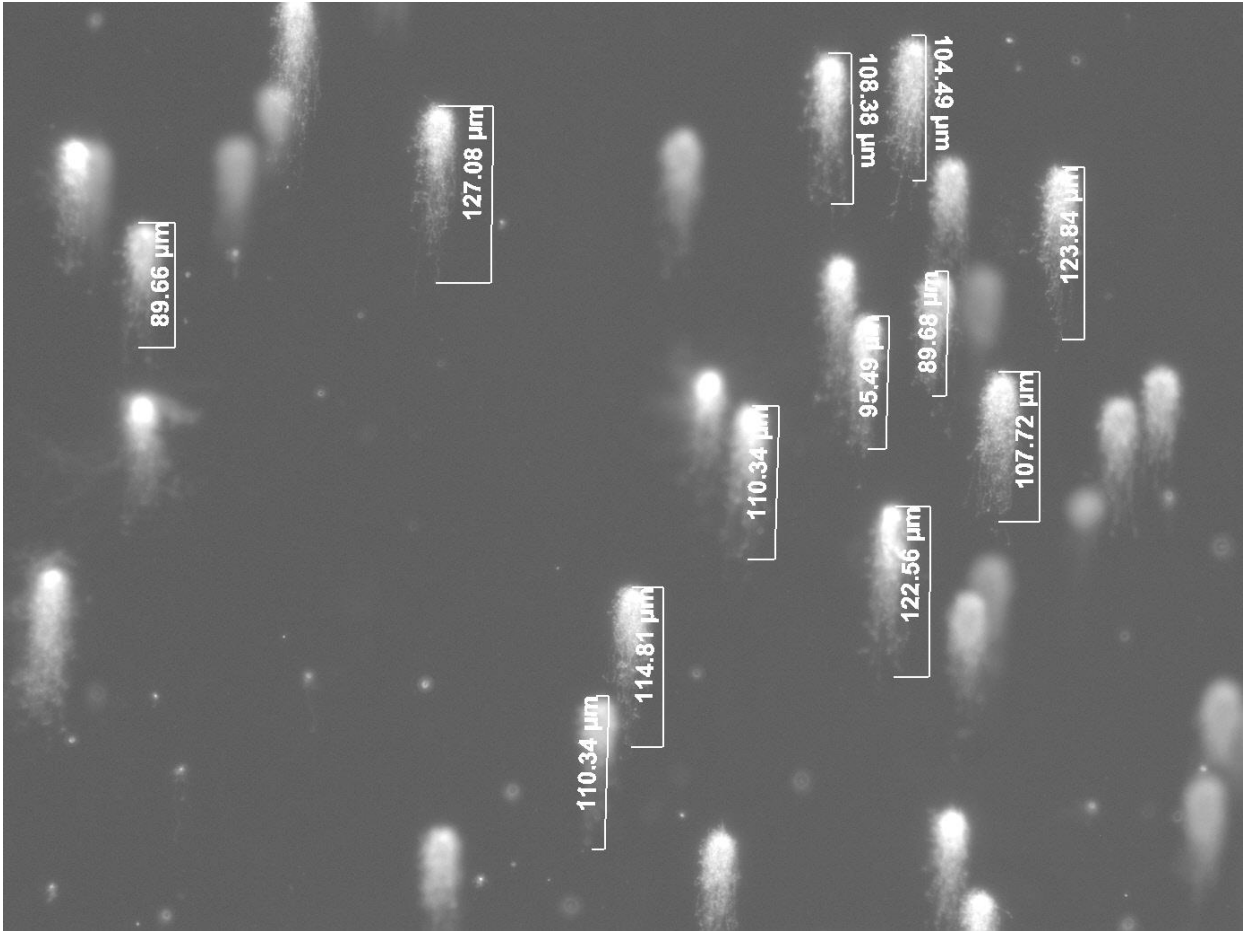


Figure 6: Comet Slide 1, Fish 4 Treatment 32 μM

Comet slide 1 has both a workable cell density and comet tails that were very in-focus. There are very few overlapping comet tails, and there are twelve tails in the plane of focus.

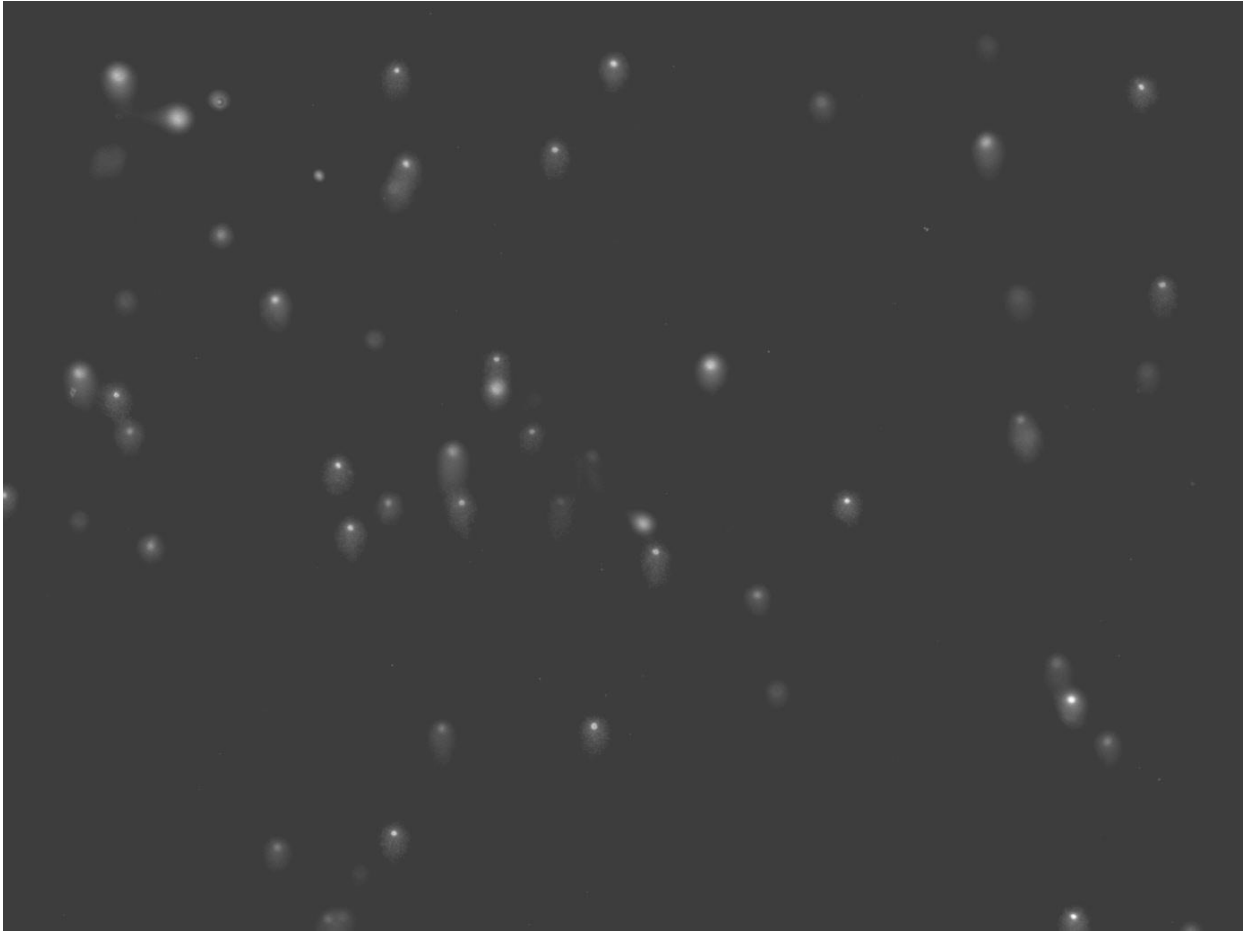


Figure 7: Comet Slide 2, Fish 3 Treatment 32 μ M

In comet slide 2, no comet tails are in the plane of focus. Therefore, none could be measured.

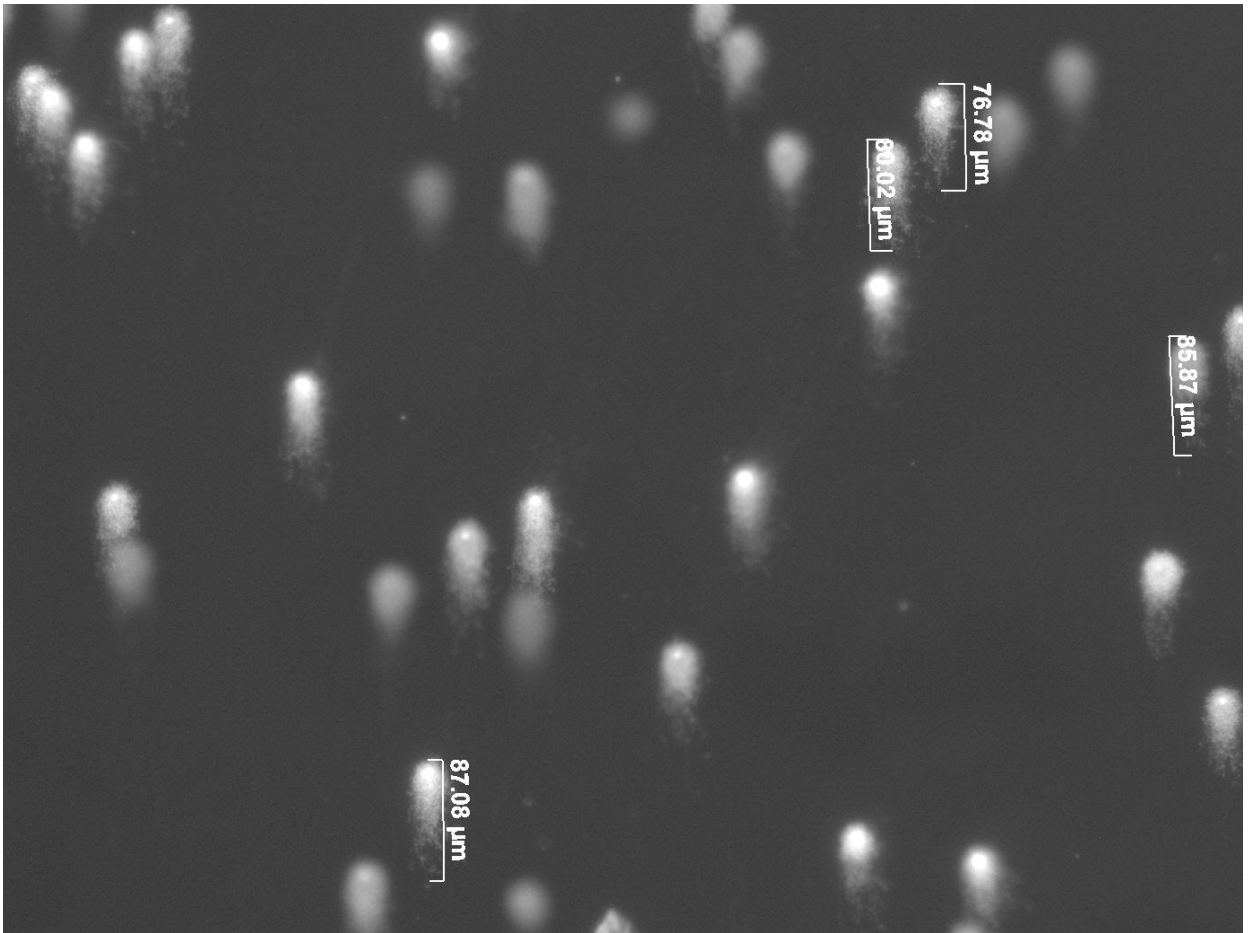


Figure 6: Comet Slide 3, Fish 4 Treatment DMSO

Comet slide 3 has only four measurable comet tails. Most of the tails are not in the plane of focus, and many of the tails that are in the pane of focus are overlapping. Due to these conditions, the tails were difficult to measure.

Appendix C: SYBR-14/PI Flow Cytometry Data

This appendix contains the flow cytometry data for the SYBR-14/PI cytotoxicity assay. Each image gives the actual number of live or dead/dying cells per each treatment group. Cells colored green or labeled SYBR-14 are living. Cells labeled PI or SYBR-14/PI are considered dead or dying.

Table 6: Fish identification key for SYBR-14/PI Assay

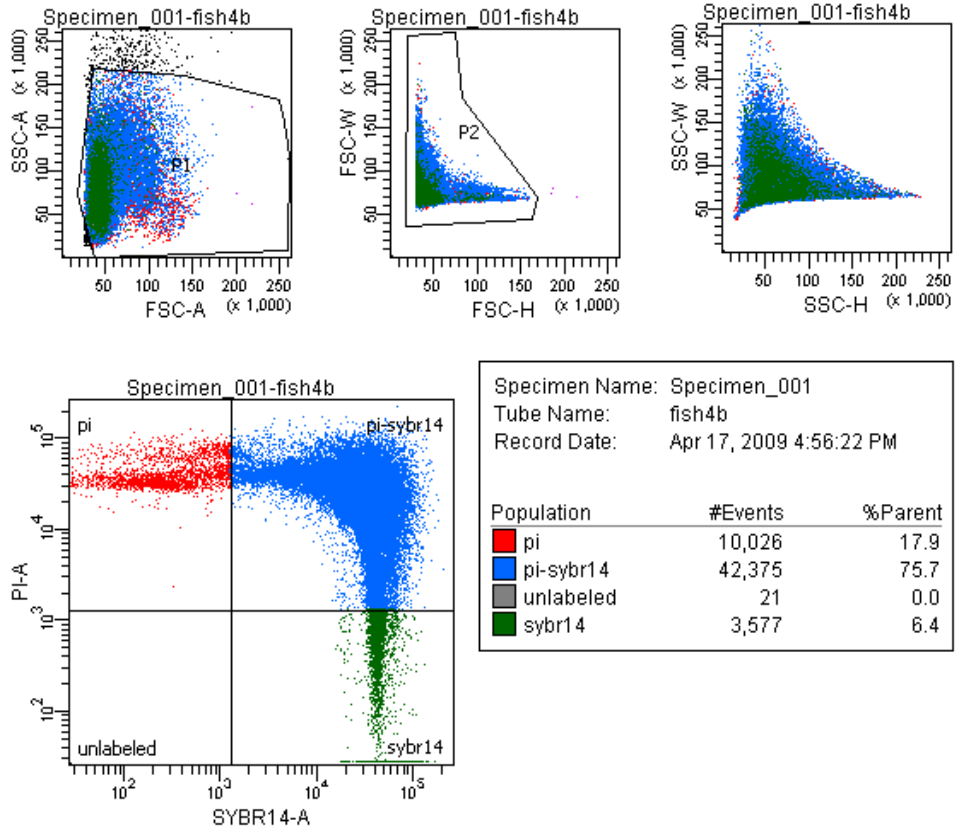
Picture Number	Tube Name (Fish Number)	Treatment
1	4b	H ₂ O ₂
2	4c	32 μM
3	4d	500 μM
4	5a	DMSO
5	5b	H ₂ O ₂
6	5c	32 μM
7	5d	500 μM
8	6a	DMSO
9	6b	H ₂ O ₂
10	6c	32 μM
11	6d	500 μM
12	8a	None
13	8b	DMSO
14	8c	H ₂ O ₂
15	8d	32 μM
16	9a	None
17	9b	DMSO
18	9c	H ₂ O ₂
19	9d	32 μM

20	7e	500 μ M
21	8e	500 μ M
22	8e	500 μ M
23	9e	500 μ M
24	9e	500 μ M

Treatment: H₂O₂

FACSDiva Version 6.1.2

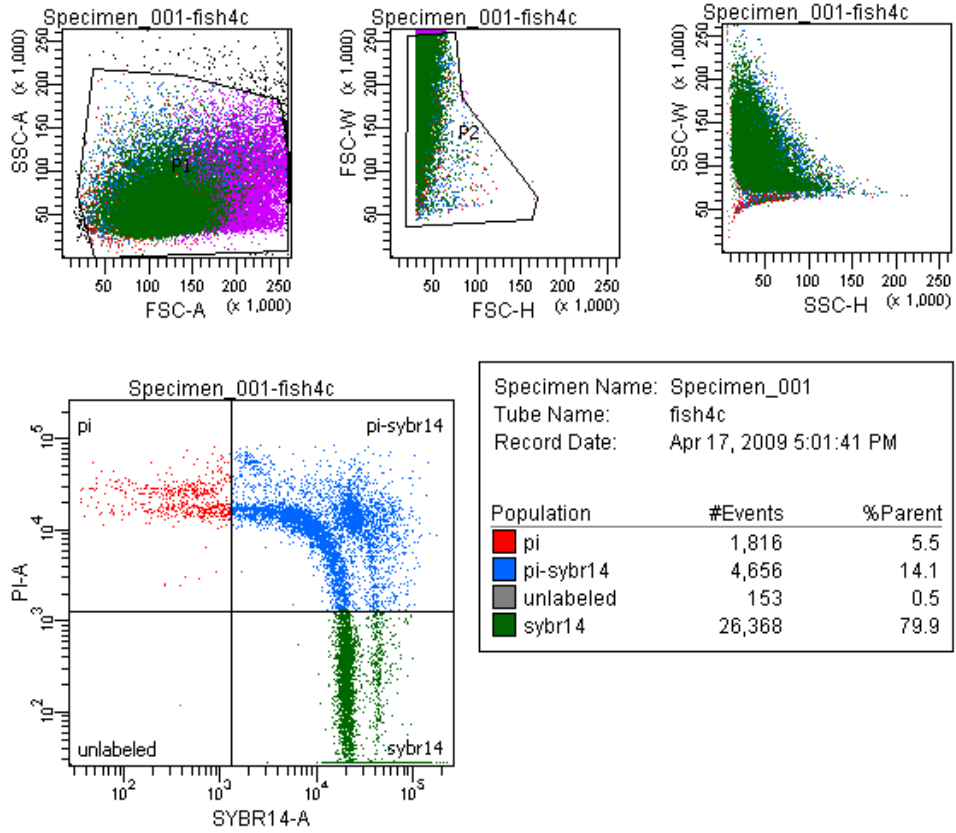
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:27 EDT



Treatment: 32 μ M

FACSDiva Version 6.1.2

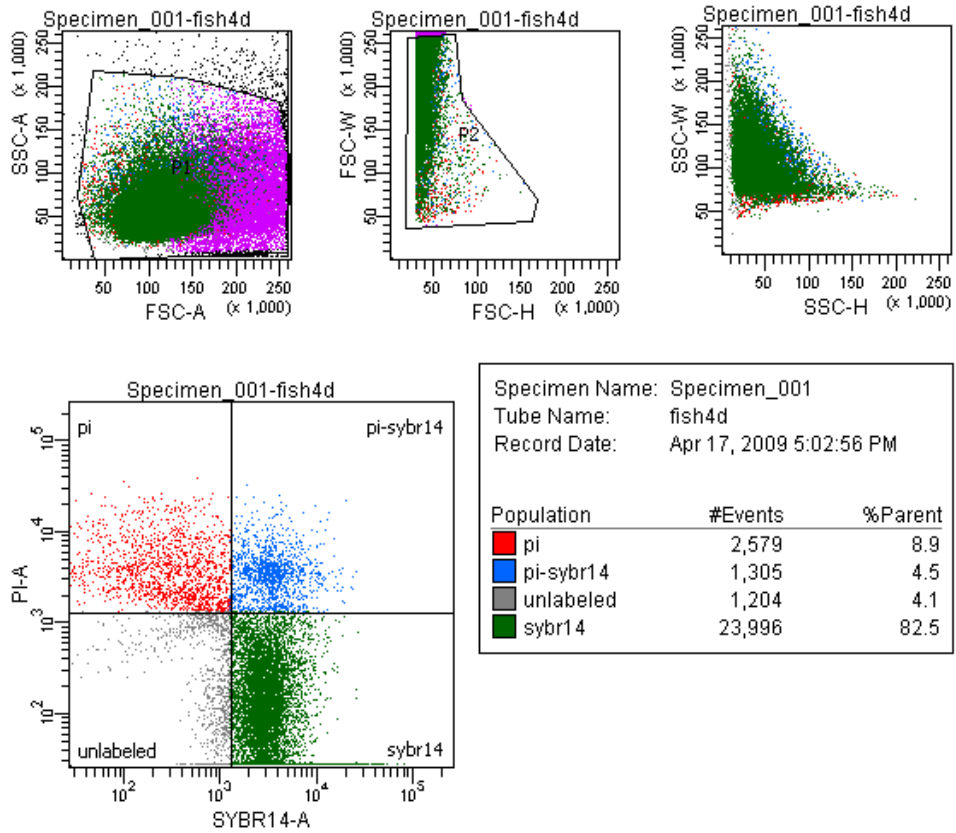
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:34 EDT



Treatment: 500 μ M

FACSDiva Version 6.1.2

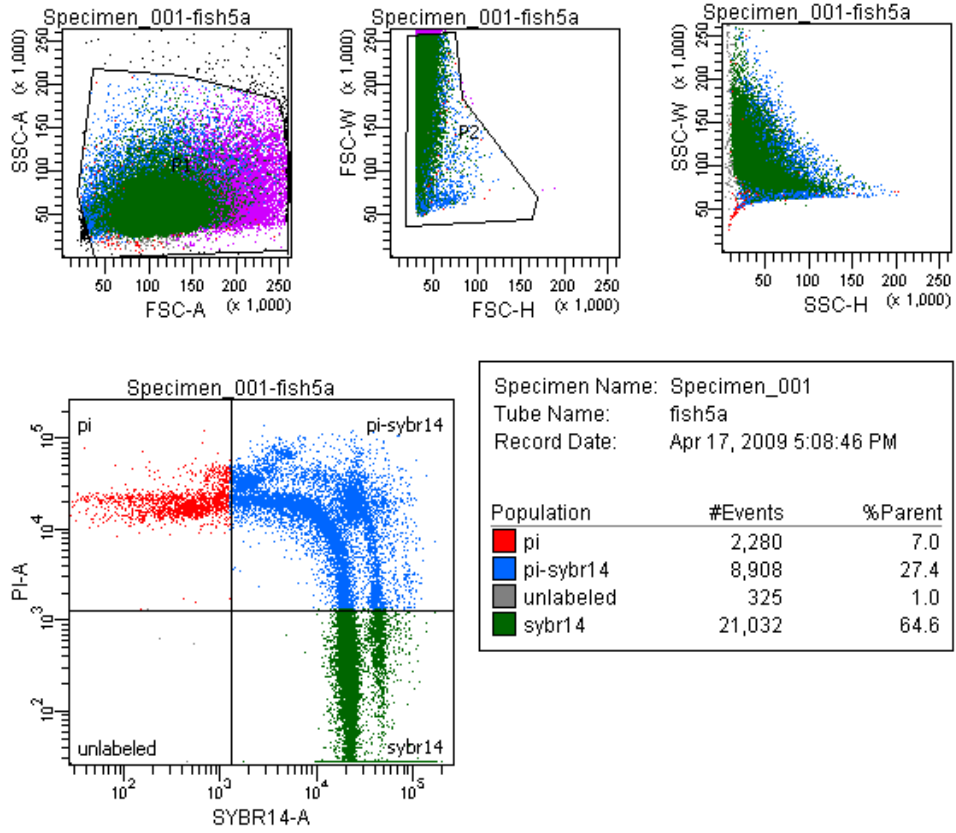
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:40 EDT



Treatment: DMSO

FACSDiva Version 6.1.2

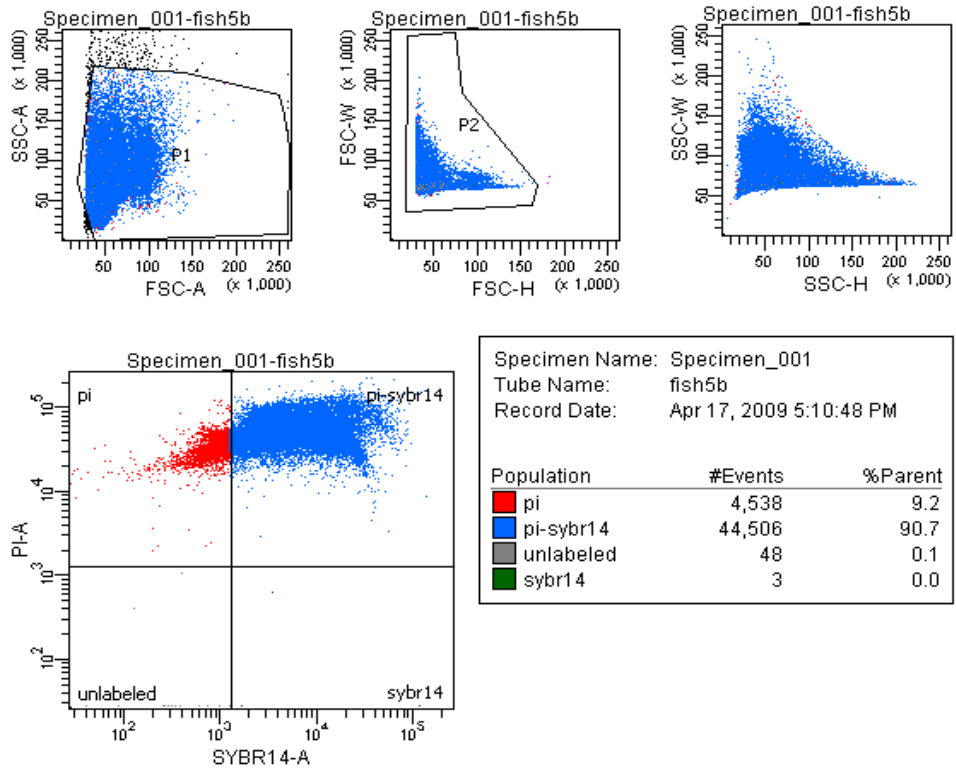
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:45 EDT



Treatment: H₂O₂

FACSDiva Version 6.1.2

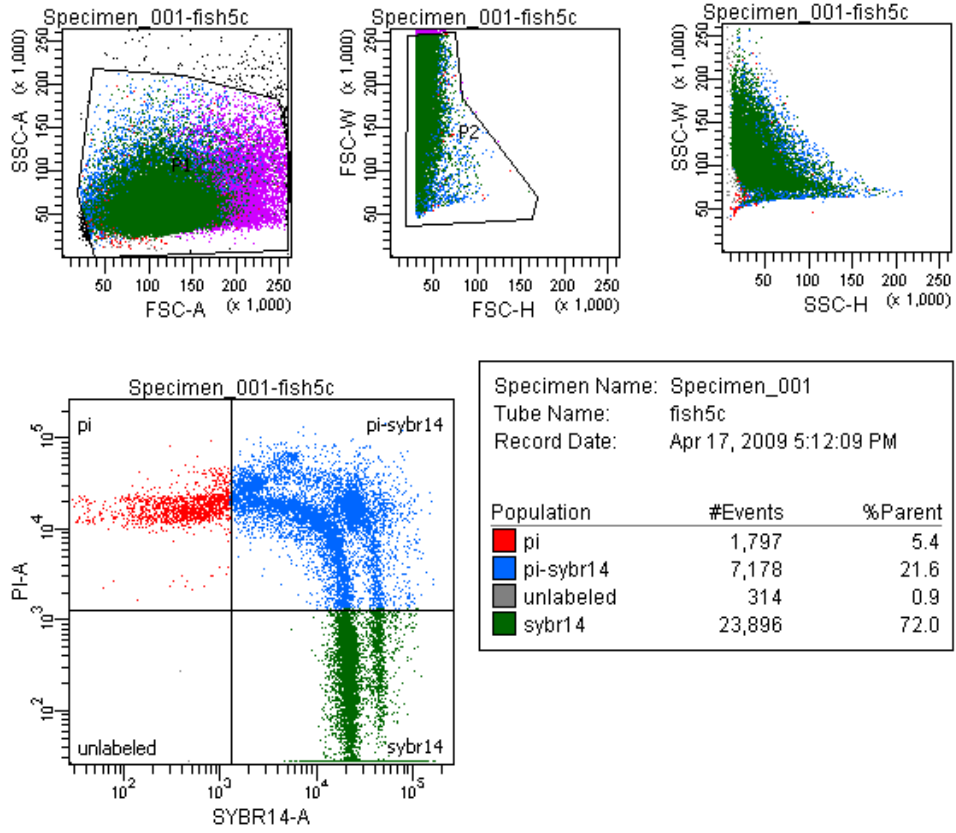
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:49 EDT



Treatment: 32 μ M

FACSDiva Version 6.1.2

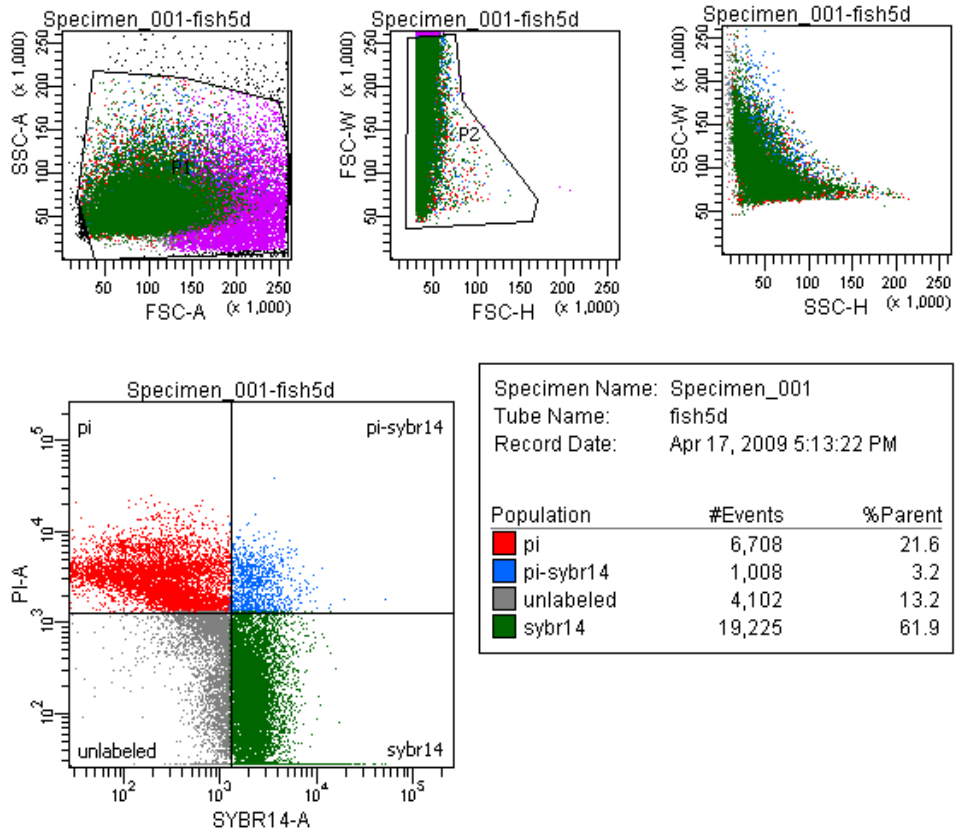
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:54 EDT



Treatment: 500 μ M

FACSDiva Version 6.1.2

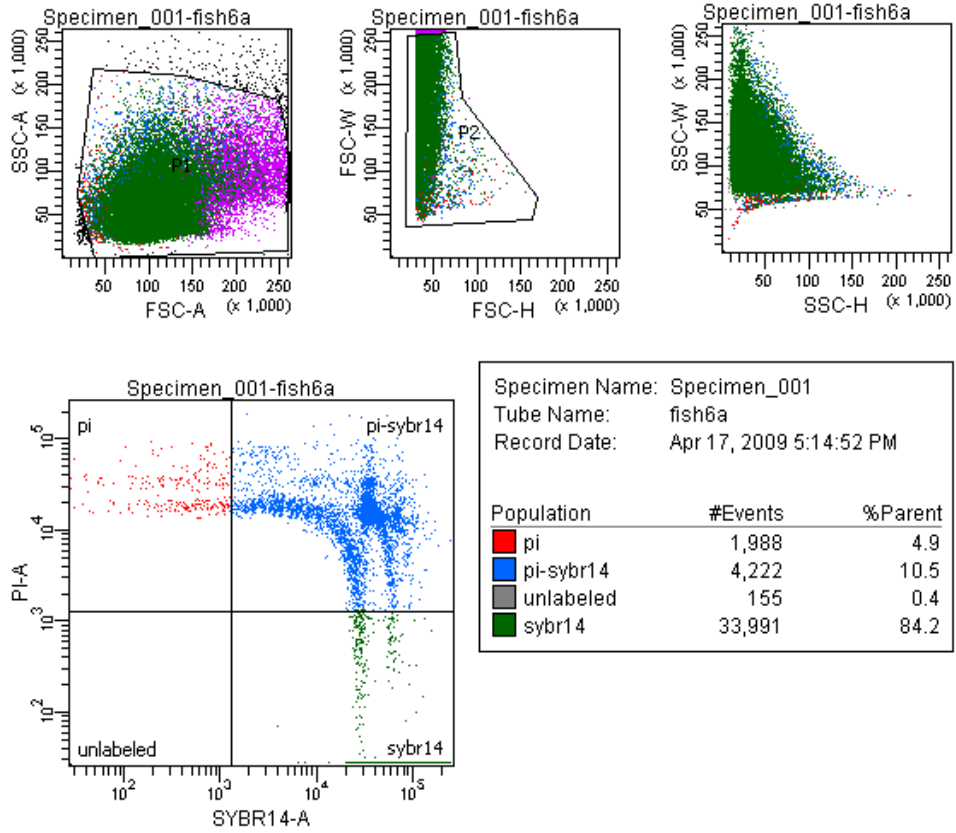
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:22:59 EDT



Treatment: DMSO

FACSDiva Version 6.1.2

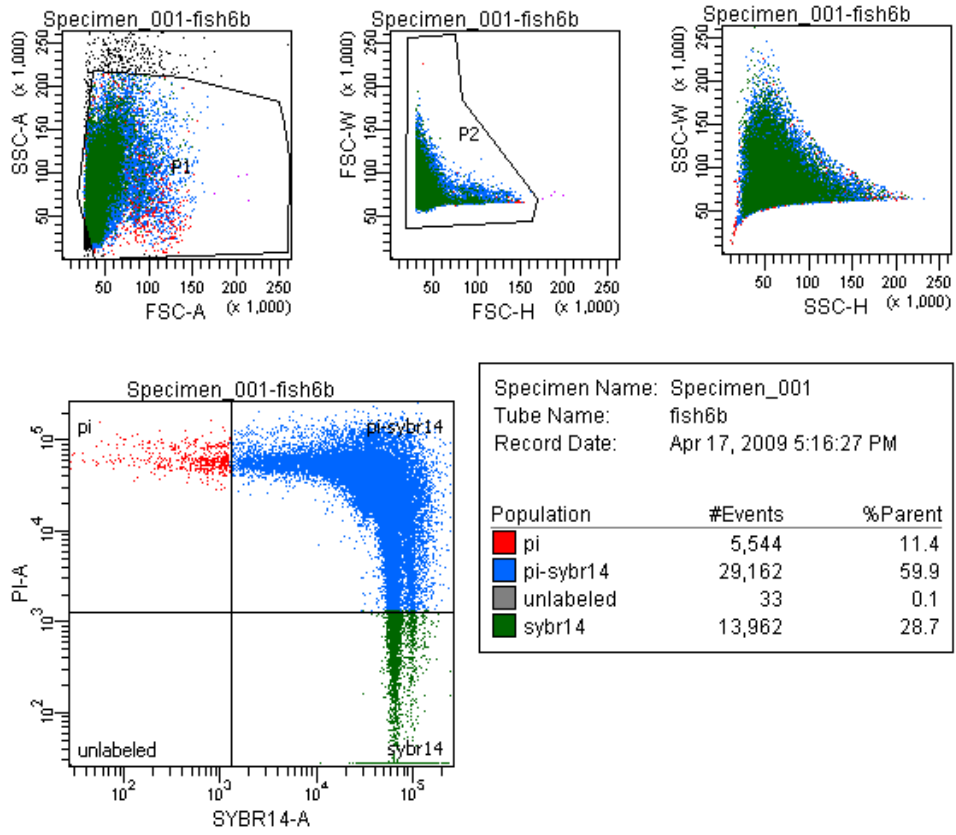
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:23:05 EDT



Treatment: H₂O₂

FACSDiva Version 6.1.2

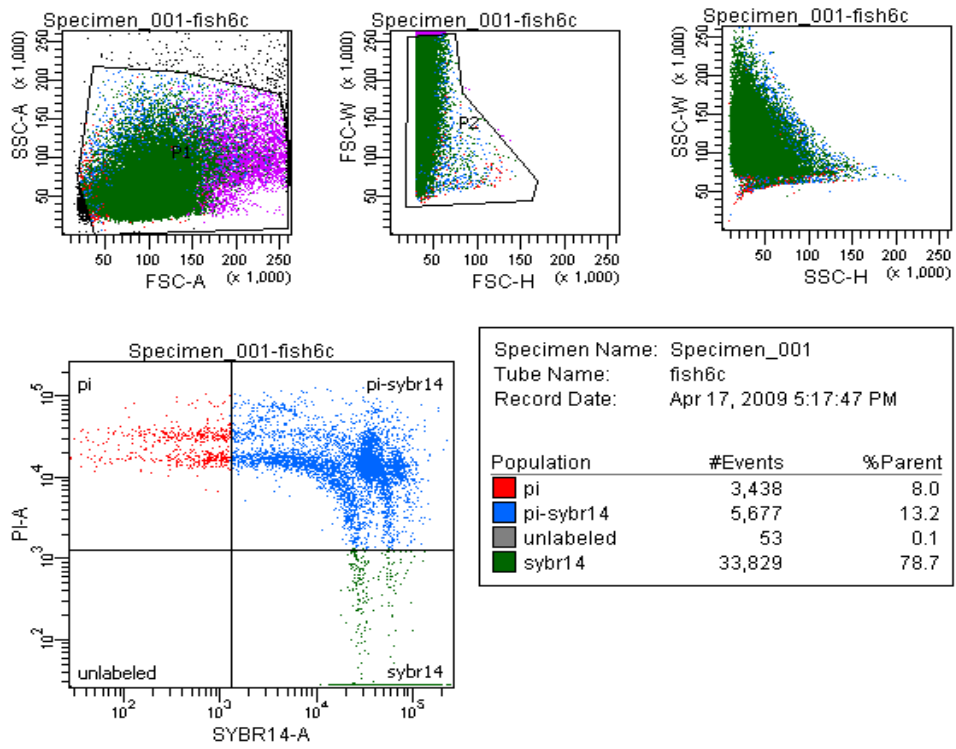
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:23:09 EDT



Treatment: 32 μ M

FACSDiva Version 6.1.2

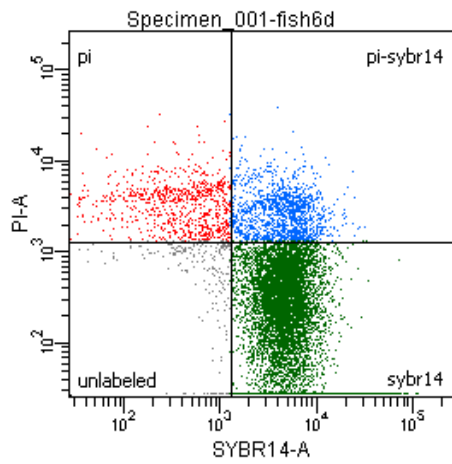
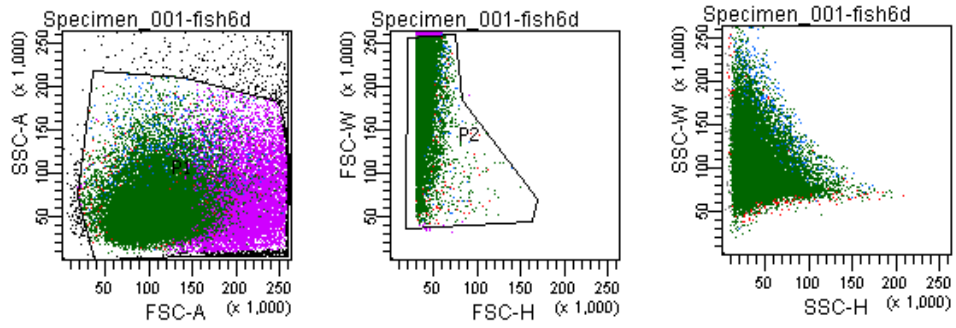
Global Sheet1 Page 1 of 1 Printed on: Fri Apr 17, 2009 05:23:15 EDT



Treatment: 500 μ M

FACSDiva Version 6.1.2

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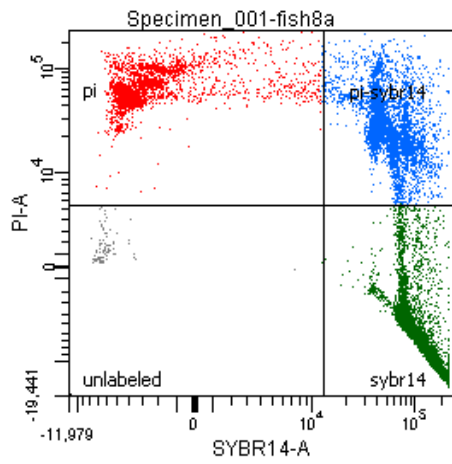
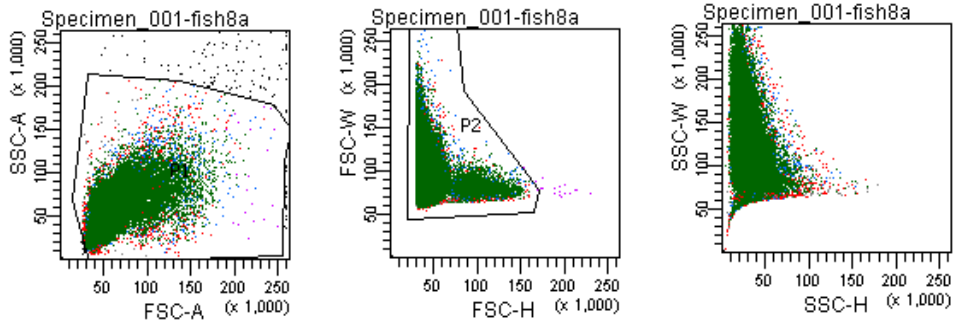


Specimen Name:	Specimen_001	
Tube Name:	fish6d	
Record Date:	Apr 17, 2009 5:18:43 PM	
Population	#Events	%Parent
pi	1,557	6.5
pi-sybr14	1,401	5.9
unlabeled	321	1.3
sybr14	20,648	86.3

Treatment: None

FACSDiva Version 6.1.2

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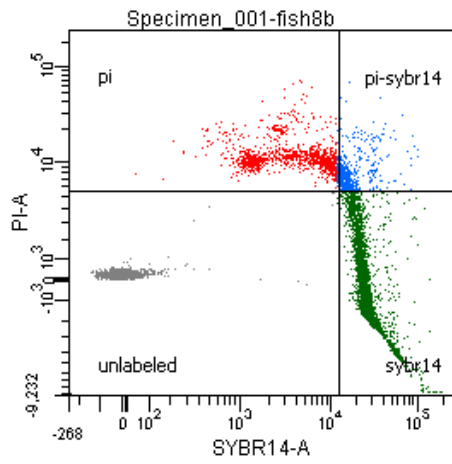
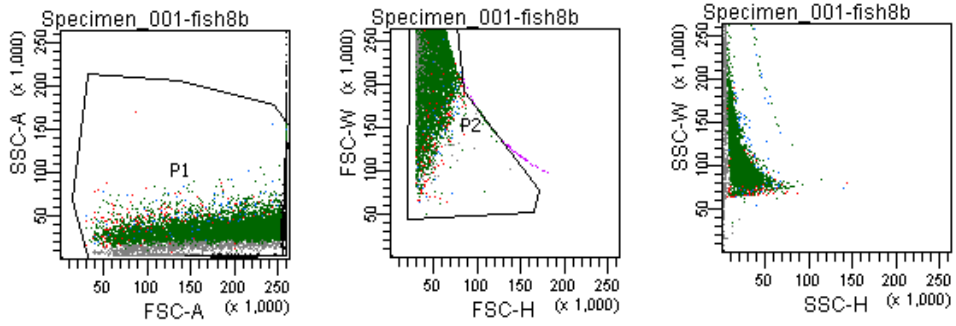
Specimen Name: Specimen_001
Tube Name: fish8a
Record Date: Apr 21, 2009 4:52:54 PM

Population	#Events	%Parent
pi	3,510	7.1
pi-sybr14	4,691	9.5
unlabeled	93	0.2
sybr14	41,278	83.3

Treatment: DMSO

FACSDiva Version 6.1.2

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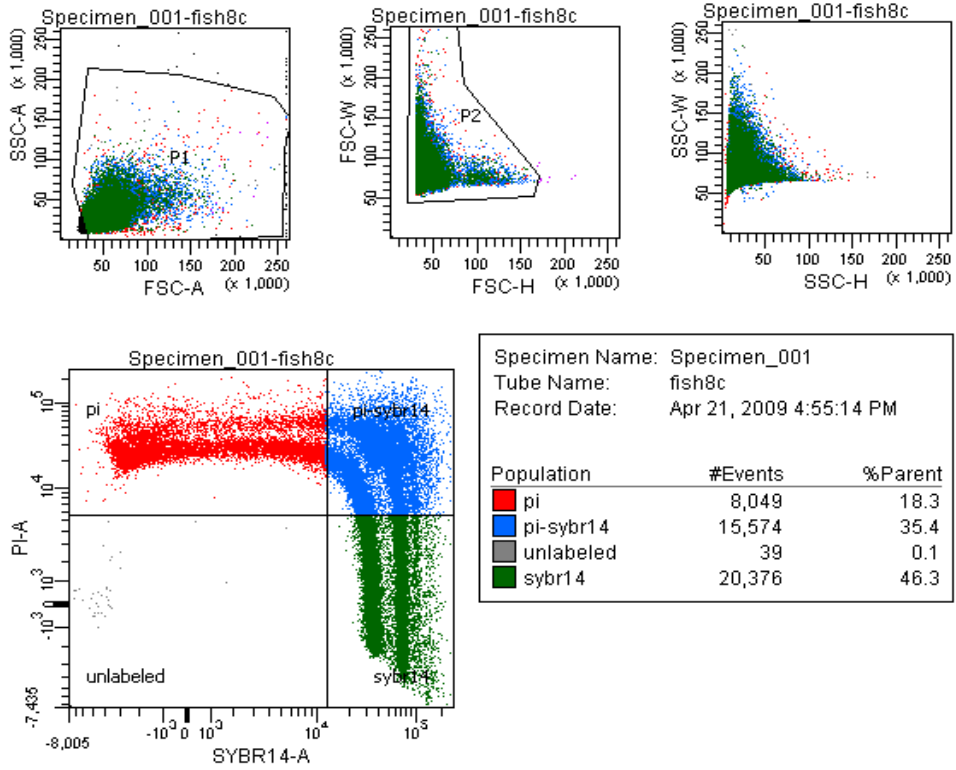


Specimen Name: Specimen_001
Tube Name: fish8b
Record Date: Apr 21, 2009 4:53:35 PM

Population	#Events	%Parent
pi	1,128	11.1
pi-sybr14	515	5.1
unlabeled	3,053	30.0
sybr14	5,469	53.8

FACSDiva Version 6.1.2

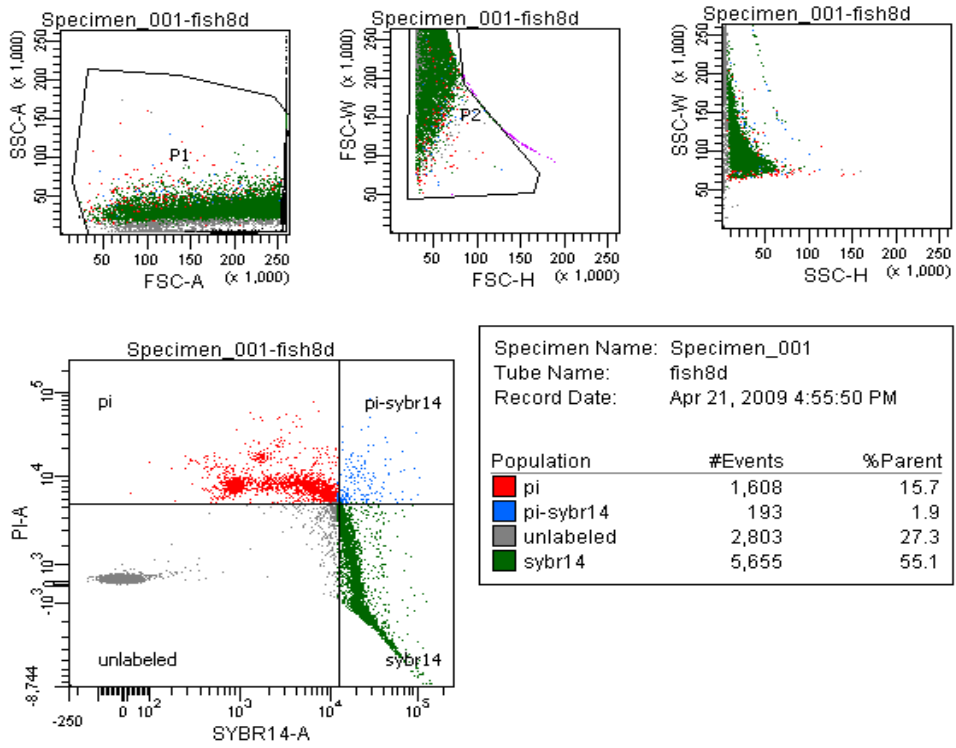
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:49:41 EDT



Treatment: 32 μ M

FACSDiva Version 6.1.2

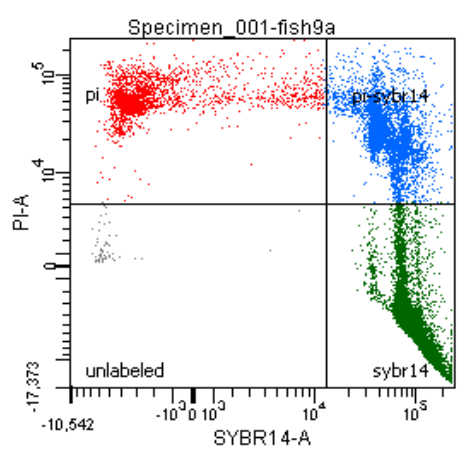
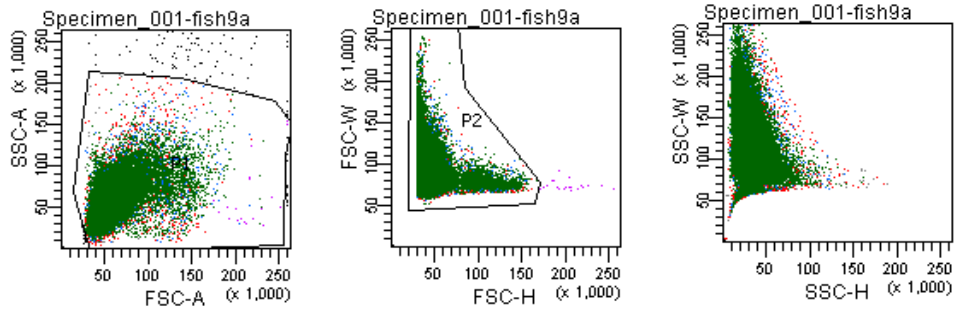
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:49:45 EDT



Treatment: None

FACSDiva Version 6.1.2

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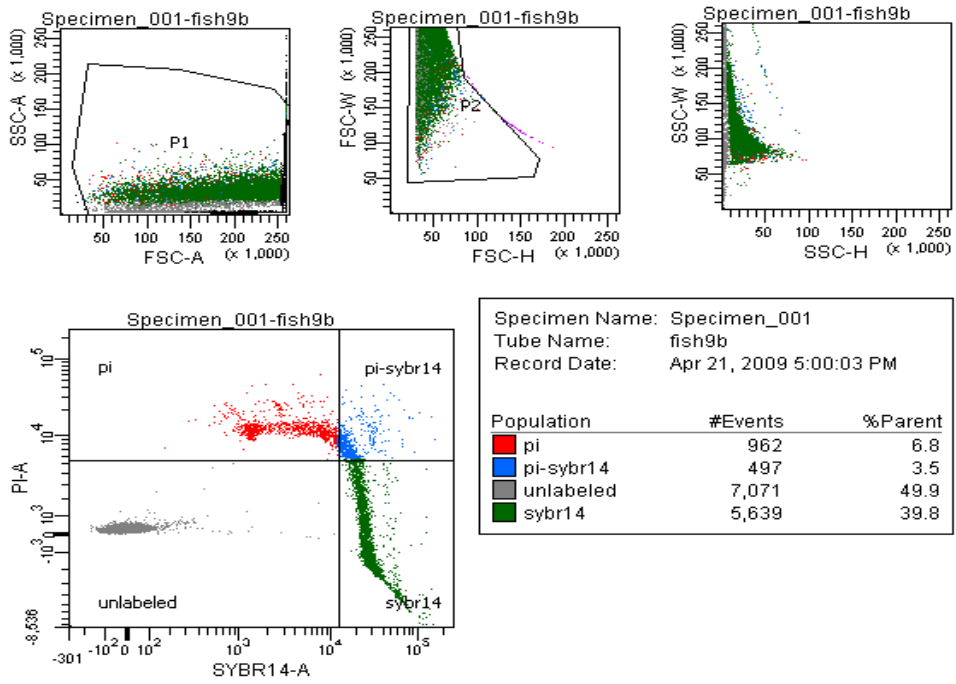


Specimen Name: Specimen_001		
Tube Name: fish9a		
Record Date: Apr 21, 2009 4:59:18 PM		
Population	#Events	%Parent
pi	3,127	6.3
pi-sybr14	4,555	9.2
unlabeled	58	0.1
sybr14	41,917	84.4

Treatment: DMSO

FACSDiva Version 6.1.2

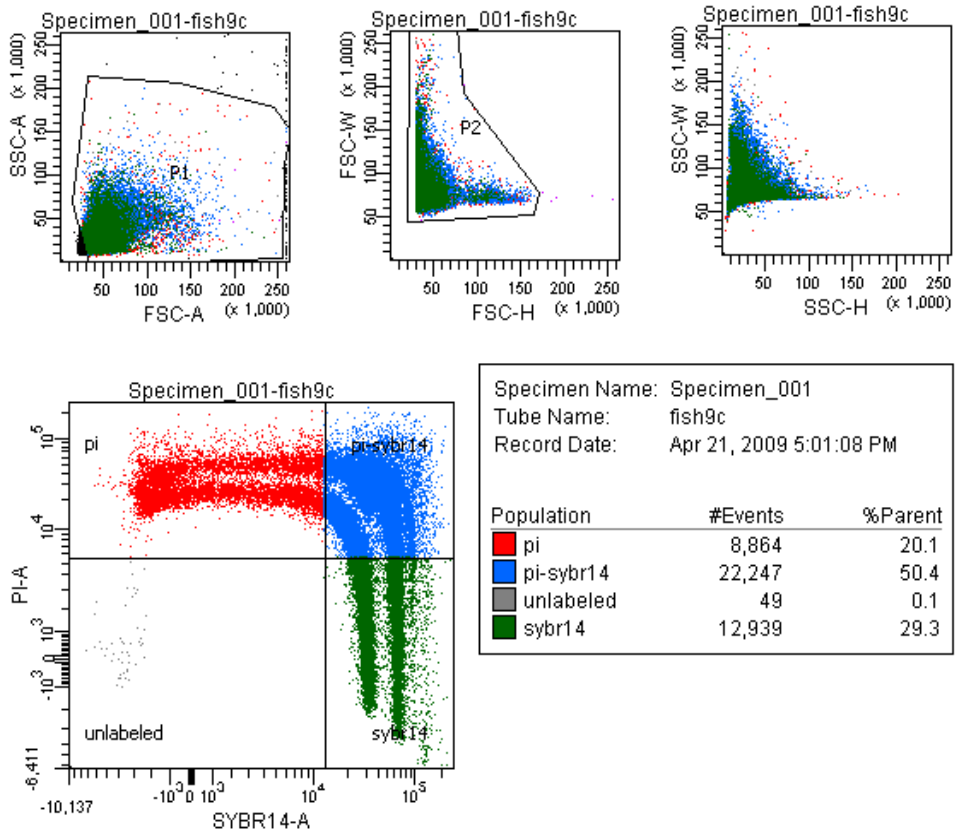
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:49:59 EDT



Treatment: H₂O₂

FACSDiva Version 6.1.2

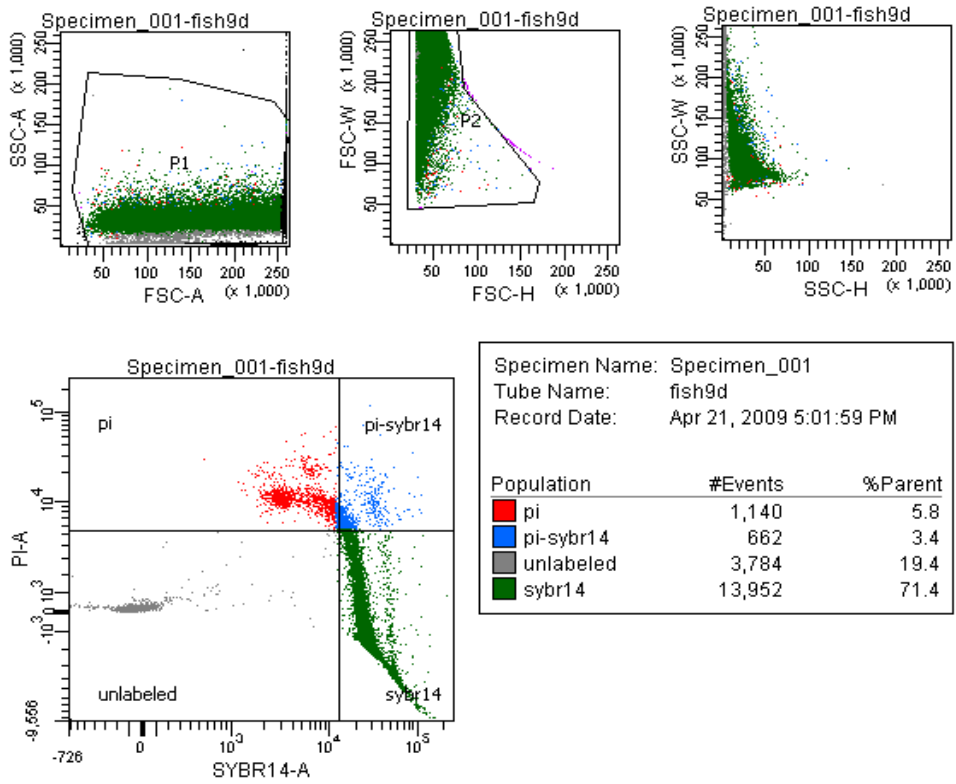
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:50:03 EDT



Treatment: 32 μ M

FACSDiva Version 6.1.2

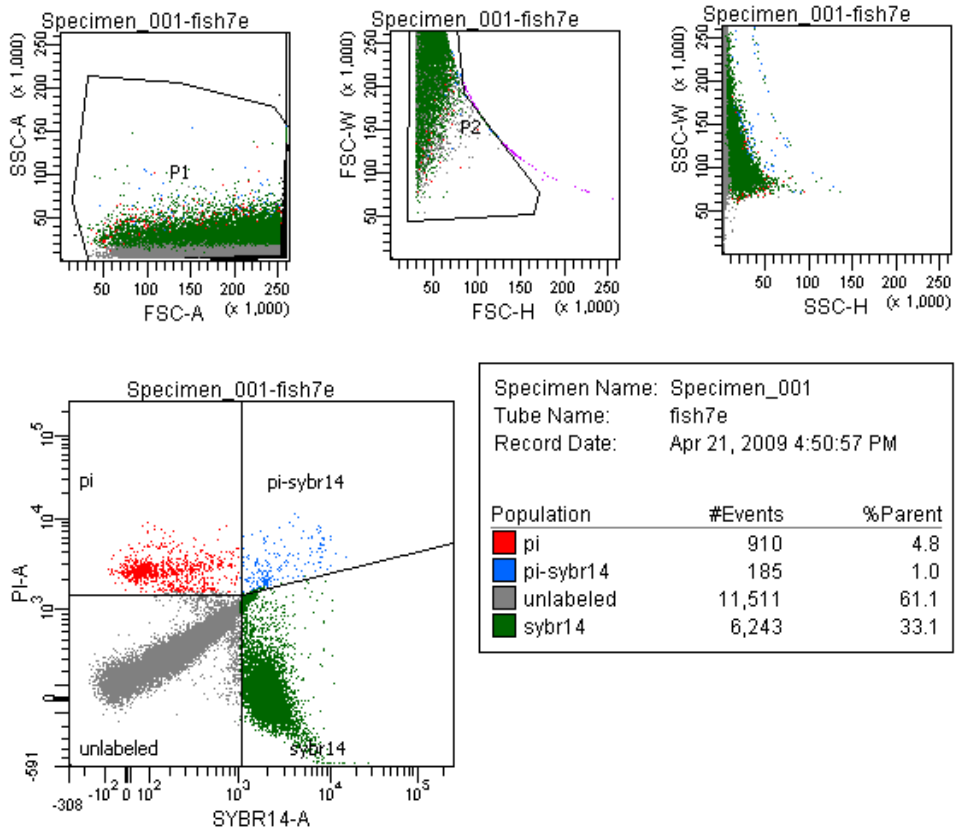
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:50:07 EDT



Treatment: 500 μ M

FACSDiva Version 6.1.2

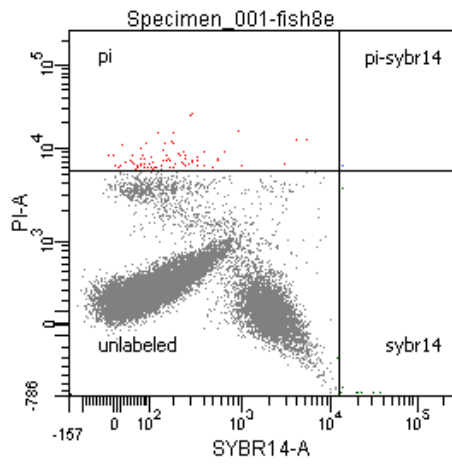
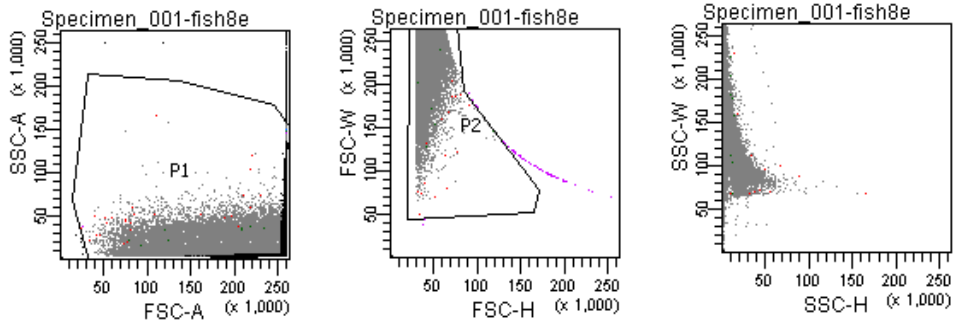
Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:51:42 EDT



Treatment: 500 μ M (Calibration 1)

FACSDiva Version 6.1.2

Global Sheet1 Page 1 of 1 Printed on: Tue Apr 21, 2009 05:49:49 EDT

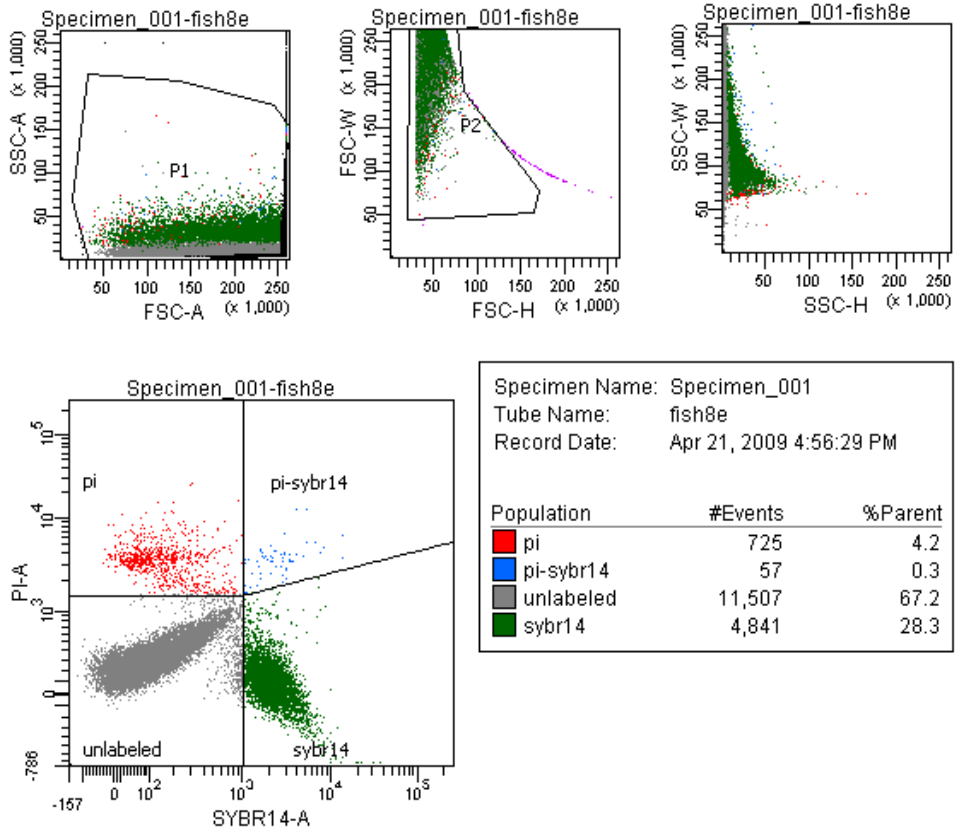


Specimen Name: Specimen_001		
Tube Name: fish8e		
Record Date: Apr 21, 2009 4:56:29 PM		
Population	#Events	%Parent
pi	76	0.4
pi-sybr14	1	0.0
unlabeled	17,044	99.5
sybr14	9	0.1

Treatment: 500 μ M (Calibration 2)

FACSDiva Version 6.1.2

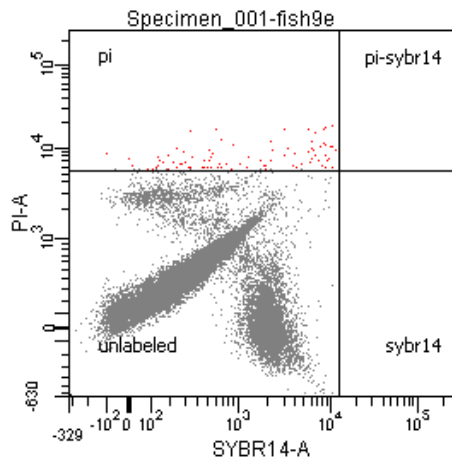
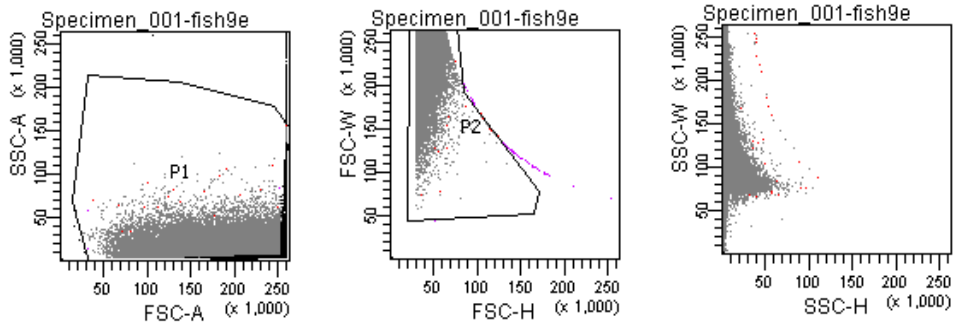
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Treatment: 500 μ M (Calibration 1)

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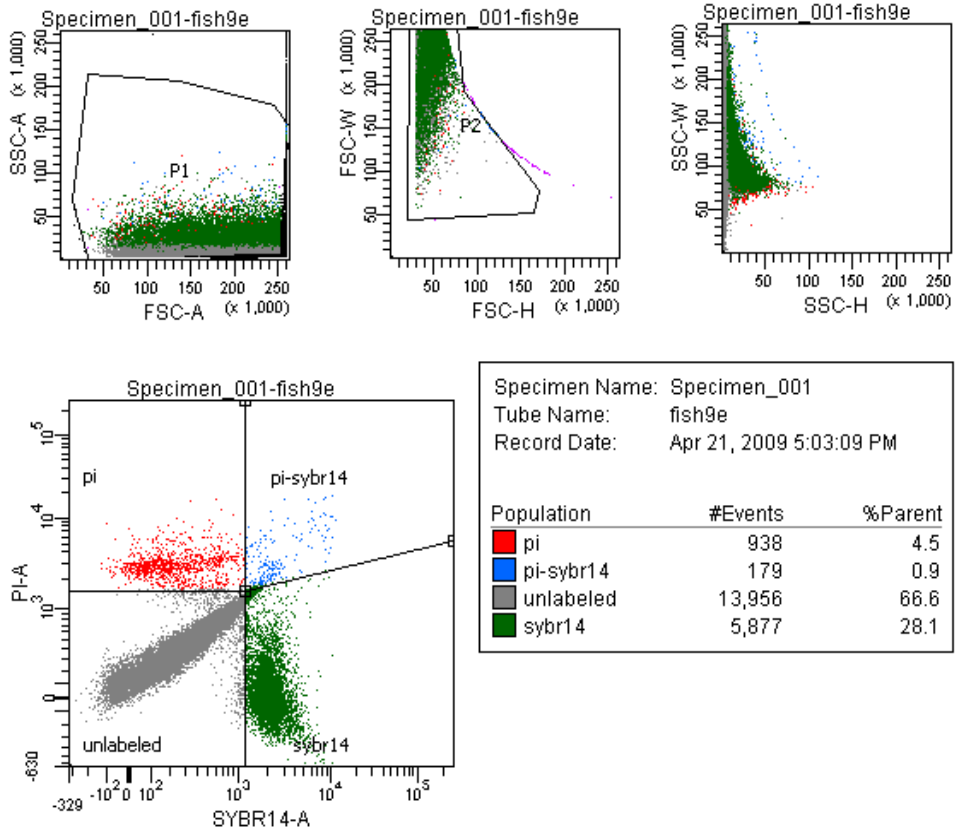


Specimen Name: Specimen_001		
Tube Name: fish9e		
Record Date: Apr 21, 2009 5:03:09 PM		
Population	#Events	%Parent
pi	84	0.4
pi-sybr14	0	0.0
unlabeled	20,866	99.6
sybr14	0	0.0

Treatment: 500 μ M (Calibration 2)

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Appendix D: Comet Data

This is a sample of the raw data we used to run SAS.

The following table contains data from fish 4, 5 and 6.

Experiment Date	Fish	A/B	treatment	expr unit	measurement date	measurerer	cell	Comet length (μM)
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	1	108.36
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	2	92.96
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	3	77.41
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	4	90.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	5	104.59
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	6	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	7	87.1
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	8	72.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	9	96.76
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	10	98.74
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	11	92.94
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	12	80.63
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	13	87.72
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	14	84.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	15	83.86
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	16	93.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	17	89.66
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	18	92.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	19	82.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	20	79.34
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	21	94.82
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	22	90.31
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	23	92.38
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	24	96.11
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	25	98.7
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	26	95.51
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	27	100.08
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	28	97.81
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	29	92.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	30	99.97
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	31	90.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	32	93.61
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	33	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	34	90.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	35	95.31
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	36	85.16
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	37	83.87
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	38	91.65
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	39	85.79
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	40	82.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	41	83.85
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	42	82.57
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	43	105.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	44	90.97
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	45	84.5
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	46	78.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	47	76.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	48	100.63
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	49	98.72
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	50	103.9

4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	51	94.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	52	105.8
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	53	92.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	54	122.01
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	55	94.87
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	56	102.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	57	92.24
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	58	89.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	59	100.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	60	104.49
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	61	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	62	114.82
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	63	94.83
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	64	99.33
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	65	127.75
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	66	74.83
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	67	109.01
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	68	103.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	69	72.96
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	70	95.51
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	71	104.54
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	72	109.65
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	73	111.59
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	74	96.75
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	75	110.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	76	93.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	77	84.5
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	78	101.32
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	79	110.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	80	118.1
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	81	120.62
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	82	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	83	77.41
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	84	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	85	84.52
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	86	83.2
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	87	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	88	83.21
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	89	101.37
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	90	92.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	91	84.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	92	72.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	93	81.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	94	85.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	95	89.05
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	96	80.64
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	97	92.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	98	84.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	99	72.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	100	81.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	101	85.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	102	89.05
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	103	80.64
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	104	108.36
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	105	92.96
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	106	77.41
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	107	90.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	108	104.59
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	109	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	110	87.1
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	111	72.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	112	96.76
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	113	98.74

4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	114	92.94
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	115	80.63
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	116	87.72
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	117	84.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	118	83.86
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	119	93.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	120	89.66
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	121	92.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	122	82.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	123	79.34
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	124	94.82
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	125	90.31
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	126	92.38
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	127	96.11
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	128	98.7
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	129	95.51
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	130	100.08
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	131	97.81
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	132	92.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	133	99.97
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	134	90.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	135	93.61
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	136	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	137	90.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	138	95.31
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	139	85.16
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	140	83.87
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	141	91.65
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	142	85.79
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4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	145	82.57
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	146	105.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	147	90.97
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	148	84.5
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	149	78.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	150	76.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	151	100.63
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	152	98.72
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	153	103.9
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	154	94.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	155	105.8
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	156	92.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	157	122.01
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	158	94.87
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	159	102.56
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	160	92.24
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	161	89.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	162	100.69
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	163	104.49
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	164	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	165	114.82
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	166	94.83
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	167	99.33
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	168	127.75
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	169	74.83
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	170	109.01
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	171	103.23
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	172	72.96
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	173	95.51
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	174	104.54
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	175	109.65
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	176	111.59

4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	177	96.75
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	178	110.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	179	93.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	180	84.5
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	181	101.32
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	182	110.3
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	183	118.1
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	184	120.62
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	185	101.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	186	77.41
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	187	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	188	84.52
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	189	83.2
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	190	105.78
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	191	83.21
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	192	101.37
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	193	92.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	194	84.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	195	72.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	196	81.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	197	85.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	198	89.05
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	199	80.64
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	200	92.29
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	201	84.53
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	202	72.27
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	203	81.92
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	204	85.15
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	205	89.05
4/17/2009	4	A	DMSO	1	7/16/2009	Mickey/Brad	206	80.64
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	207	103.33
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	208	84.53
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	209	103.07
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	210	79.36
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	211	98.07
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	212	105.83
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	213	89.03
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	214	117.43
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	215	99.5
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	216	91.61
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	217	111.63
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	218	82.57
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	219	76.8
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	220	98.69
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	221	75.69
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	222	90.63
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	223	90.31
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	224	97.4
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	225	75.48
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	226	105.15
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	227	105.79
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	228	99.33
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	229	111.81
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	230	80.79
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	231	107.07
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	232	100.03
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	233	82.57
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	234	69.66
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	235	65.16
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	236	99.98
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	237	76.11
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	238	78.09
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	239	81.27

4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	240	94.82
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	241	83.21
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	242	92.88
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	243	114.23
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	244	86.44
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	245	101.91
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	246	89.66
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	247	90.95
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	248	78.05
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	249	84.56
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	250	103.86
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	251	103.88
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	252	75.47
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	253	101.32
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	254	78.69
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	255	85.79
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	256	76.8
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	257	101.37
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	258	83.85
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	259	80.02
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	260	76.78
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	261	87.08
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	262	85.87
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	263	94.85
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	264	54.92
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	265	91.67
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	266	123.33
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	267	119.47
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	268	92.88
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	269	107.14
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	270	81.31
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	271	90.32
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	272	87.72
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	273	94.82
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	274	90.98
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	275	84.5
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	276	87.84
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	277	92.23
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	278	92.92
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	279	91.59
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	280	85.78
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	281	87.72
4/17/2009	4	B	DMSO	1	7/17/2009	Mickey/Brad	282	90.98
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	283	138.04
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	284	109.02
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	285	112.89
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	286	133.52
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	287	112.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	288	112.97
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	289	101.27
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	290	118.04
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	291	108.37
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	292	103.85
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	293	127.08
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	294	89.66
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	295	108.38
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	296	104.49
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	297	123.84
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	298	107.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	299	110.34
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	300	114.81
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	301	122.56
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	302	95.49

4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	303	89.68
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	304	110.34
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	305	116.11
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	306	101.28
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	307	108.37
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	308	118.69
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	309	101.27
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	310	105.15
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	311	107.08
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	312	118.04
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	313	91.61
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	314	96.16
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	315	92.24
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	316	81.92
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	317	82.57
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	318	90.3
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	319	101.96
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	320	107.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	321	98.04
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	322	94.17
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	323	91.6
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	324	77.4
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	325	83.85
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	326	79.99
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	327	87.73
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	328	93.53
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	329	97.4
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	330	112.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	331	112.26
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	332	108.36
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	333	107.81
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	334	99.98
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	335	105.79
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	336	103.22
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	337	93.53
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	338	100.63
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	339	103.23
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	340	110.34
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	341	109.74
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	342	96.89
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	343	100.03
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	344	121.27
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	345	112.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	346	104.09
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	347	103.9
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	348	99.43
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	349	96.76
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	350	105.14
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	351	114.9
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	352	103.21
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	353	93.55
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	354	89.66
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	355	109.65
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	356	87.73
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	357	94.82
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	358	117.39
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	359	112.9
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	360	95.48
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	361	83.85
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	362	90.3
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	363	94.18
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	364	85.15
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	365	94.81

4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	366	115.47
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	367	89.68
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	368	98.69
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	369	106.43
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	370	93.53
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	371	101.32
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	372	112.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	373	105.14
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	374	100.03
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	375	117.43
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	376	101.91
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	377	116.76
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	378	98.75
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	379	96.11
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	380	107.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	381	91.59
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	382	103.2
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	383	95.46
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	384	94.89
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	385	101.37
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	386	78.07
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	387	109.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	388	105.14
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	389	108.36
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	390	107.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	391	110.94
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	392	96.76
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	393	101.28
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	394	125.14
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	395	107.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	396	100.69
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	397	101.91
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	398	106.43
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	399	114.17
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	400	121.97
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	401	110.94
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	402	100.62
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	403	115.46
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	404	101.28
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	405	94.83
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	406	102.59
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	407	110.95
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	408	92.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	409	92.23
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	410	114.86
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	411	124.55
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	412	105.83
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	413	100.63
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	414	101.92
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	415	98.72
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	416	94.89
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	417	114.84
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	418	112.88
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	419	92.24
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	420	108.43
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	421	113.24
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	422	110.95
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	423	101.28
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	424	123.24
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	425	117.39
4/17/2009	4	A	32	2	7/9/2009	Mickey/Brad	426	123.2
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	427	109.2
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	428	118.04

4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	429	127.07
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	430	109.07
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	431	117.74
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	432	111.6
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	433	98.7
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	434	113.88
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	435	120.62
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	436	102.56
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	437	97.65
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	438	80.64
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	439	105.88
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	440	98.69
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	441	98.74
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	442	85.14
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	443	89.05
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	444	79.34
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	445	107.72
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	446	85.87
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	447	77.44
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	448	86.44
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	449	87.87
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	450	95.72
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	451	96.89
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	452	93.6
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	453	105.21
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	454	111.61
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	455	96.92
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	456	98.06
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	457	121.24
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	458	94.17
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	459	93.38
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	460	98.76
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	461	93.2
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	462	110.97
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	463	99.7
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	464	90.98
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	465	90.3
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	466	134.38
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	467	121.27
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	468	105.94
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	469	119.99
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	470	81.92
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	471	86.58
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	472	98.69
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	473	132.79
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	474	89.8
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	475	94.17
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	476	105.42
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	477	89.09
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	478	100.64
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	479	96.12
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	480	71.62
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	481	115.92
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	482	106.58
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	483	94.87
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	484	82.72
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	485	83.94
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	486	107.09
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	487	107.2
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	488	94.96
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	489	98.07
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	490	81.31
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	491	85.15

4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	492	86.55
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	493	91.86
4/17/2009	4	B	32	2	7/17/2009	Mickey/Brad	494	86.55
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	495	138.69
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	496	116.77
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	497	105.14
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	498	116.1
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	499	111.63
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	500	141.9
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	501	112.9
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	502	93.55
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	503	97.41
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	504	90.49
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	505	129.06
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	506	104.88
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	507	82.65
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	508	92.25
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	509	91.74
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	510	97.7
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	511	110.48
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	512	87.78
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	513	103.22
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	514	102.56
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	515	90.31
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	516	107.95
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	517	100.63
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	518	98.21
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	519	86.84
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	520	82.65
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	521	91.22
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	522	81.27
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	523	89.07
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	524	89.77
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	525	86.44
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	526	83.24
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	527	78.09
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	528	88.45
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	529	90.3
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	530	100.05
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	531	99.33
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	532	106.43
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	533	91.06
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	534	88.42
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	535	99.43
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	536	89.01
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	537	98.69
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	538	107.07
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	539	105.79
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	540	108.39
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	541	92.99
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	542	123.85
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	543	109.65
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	544	105.17
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	545	103.85
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	546	92.94
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	547	108.38
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	548	100.69
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	549	106.43
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	550	108.39
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	551	100.62
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	552	92.89
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	553	96.11
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	554	109.1

4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	555	87.08
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	556	114.84
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	557	105.78
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	558	93.53
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	559	90.38
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	560	92.24
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	561	107.72
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	562	100.64
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	563	101.27
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	564	118.04
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	565	116.16
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	566	101.27
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	567	109.01
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	568	118.04
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	569	116.83
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	570	110.99
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	571	118.05
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	572	97.41
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	573	105.4
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	574	114.48
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	575	109.7
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	576	101.53
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	577	75.48
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	578	84.52
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	579	74.18
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	580	81.28
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	581	86.44
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	582	78.06
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	583	69.03
4/17/2009	4	A	500	3	7/16/2009	Mickey/Brad	584	57.46
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	585	105.79
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	586	94.83
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	587	99.98
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	588	88.48
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	589	101.43
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	590	93.53
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	591	121.27
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	592	89.66
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	593	89.05
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	594	90.32
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	595	81.31
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	596	67.77
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	597	96.92
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	598	87.1
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	599	81.28
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	600	98.74
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	601	80.63
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	602	92.26
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	603	93.53
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	604	92.96
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	605	88.37
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	606	87.19
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	607	88.37
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	608	92.35
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	609	84.5
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	610	94.96
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	611	96.83
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	612	112.24
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	613	120
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	614	96.89
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	615	98.05
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	616	103.86
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	617	76.11

4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	618	112.35
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	619	90.97
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	620	101.27
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	621	113.53
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	622	121.91
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	623	105.15
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	624	79.98
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	625	81.29
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	626	95.52
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	627	81.48
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	628	87.54
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	629	91.97
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	630	103.23
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	631	77.08
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	632	103.53
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	633	90.49
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	634	78.06
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	635	88.37
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	636	90.98
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	637	83.86
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	638	73.6
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	639	88.37
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	640	107.19
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	641	97.4
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	642	89.03
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	643	98.4
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	644	68.38
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	645	86.19
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	646	77.41
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	647	90.36
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	648	81.94
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	649	70.98
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	650	83.85
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	651	82.62
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	652	96.78
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	653	93.53
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	654	90.95
4/17/2009	4	B	500	3	7/17/2009	Mickey/Brad	655	71.59
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	656	98.05
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	657	101.96
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	658	96.53
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	659	98.69
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	660	91.53
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	661	85.62
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	662	97.37
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	663	100.65
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	664	105.47
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	665	91.63
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	666	101.4
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	667	102.57
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	668	119.02
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	669	89.03
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	670	93.2
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	671	87.19
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	672	96.66
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	673	83.56
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	674	83.56
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	675	96.78
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	676	94.92
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	677	92.27
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	678	98.74
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	679	77.41
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	680	89.01

4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	681	86.55
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	682	89.68
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	683	84.5
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	684	87.84
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	685	86.45
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	686	97.4
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	687	88.37
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	688	91.09
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	689	83.45
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	690	71.14
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	691	79.98
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	692	87.08
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	693	90.3
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	694	95.46
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	695	81.27
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	696	87.73
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	697	79.99
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	698	102.16
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	699	102.65
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	700	100.65
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	701	109.66
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	702	82.65
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	703	83.33
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	704	98.72
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	705	89.03
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	706	88.37
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	707	76.8
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	708	81.27
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	709	81.27
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	710	89.16
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	711	99.36
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	712	99.99
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	713	87.74
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	714	107.91
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	715	105.81
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	716	86.58
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	717	100.79
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	718	97.4
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	719	114.39
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	720	105.94
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	721	110.95
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	722	101.37
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	723	107.08
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	724	79.99
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	725	94.81
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	726	94.17
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	727	91.61
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	728	106.46
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	729	89.69
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	730	92.24
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	731	81.93
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	732	82.58
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	733	93.53
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	734	105.14
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	735	92.88
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	736	98.69
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	737	101.91
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	738	105.79
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	739	103.2
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	740	106.47
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	741	76.11
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	742	82.56
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	743	82.58

4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	744	81.98
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	745	64.5
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	746	69.69
4/17/2009	4	A	H ₂ O ₂	4	7/16/2009	Mickey/Brad	747	68.37
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	748	115.47
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	749	121.29
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	750	116.12
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	751	112.25
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	752	116.75
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	753	108.37
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	754	101.91
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	755	70.96
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	756	101.93
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	757	99.33
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	758	126.42
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	759	105.94
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	760	127.07
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	761	116.75
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	762	94.17
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	763	116.13
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	764	103.9
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	765	110.3
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	766	107.75
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	767	98.69
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	768	92.89
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	769	119.35
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	770	104.52
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	771	119.34
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	772	99.36
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	773	100.67
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	774	110.31
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	775	102.56
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	776	95.46
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	777	115.46
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	778	94.82
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	779	119.33
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	780	110.97
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	781	105.78
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	782	112.88
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	783	105.78
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	784	107.72
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	785	90.3
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	786	108.36
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	787	103.85
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	788	119.97
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	789	90.31
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	790	85.14
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	791	101.91
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	792	123.88
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	793	106.43
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	794	97.4
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	795	94.17
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	796	113.52
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	797	98.69
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	798	93.53
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	799	103.2
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	800	104.49
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	801	87.73
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	802	118.7
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	803	101.92
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	804	102.59
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	805	92.94
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	806	120.03

4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	807	69.66
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	808	105.17
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	809	134.17
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	810	95.46
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	811	102.57
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	812	110.99
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	813	98.04
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	814	87.1
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	815	108.45
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	816	101.91
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	817	127.71
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	818	110.3
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	819	142.55
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	820	96.75
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	821	94.17
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	822	96.11
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	823	134.2
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	824	129.69
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	825	102.56
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	826	112.3
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	827	102.56
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	828	106.66
4/17/2009	4	B	H ₂ O ₂	4	7/17/2009	Mickey/Brad	829	112.88
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	830	97.11
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	831	90.98
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	832	123.24
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	833	84.48
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	834	63.22
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	835	95.57
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	836	95.19
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	837	114.84
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	838	70.32
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	839	117.79
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	840	91.09
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	841	87.1
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	842	85.94
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	843	95.08
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	844	89.02
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	845	82.92
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	846	90.31
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	847	84.62
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	848	88.52
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	849	88.45
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	850	86.44
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	851	71.19
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	852	96.16
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	853	81.29
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	854	66.46
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	855	77.62
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	856	82.62
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	857	87.11
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	858	98.74
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	859	120.18
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	860	119.44
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	861	98.18
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	862	92.88
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	863	103.88
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	864	114.35
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	865	117.42
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	866	117.57
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	867	130.01
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	868	124.17
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	869	110.3

4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	870	89.66
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	871	111.9
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	872	89.66
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	873	95.68
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	874	125.21
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	875	117.3
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	876	88.12
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	877	83.29
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	878	107.23
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	879	97.01
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	880	94.81
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	881	96.21
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	882	88.12
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	883	83.29
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	884	107.23
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	885	97.01
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	886	94.81
4/17/2009	5	A	DMSO	5	7/17/2009	Mickey/Brad	887	96.21
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	888	99.98
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	889	94.21
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	890	99.99
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	891	130.3
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	892	102.56
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	893	76.75
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	894	73.58
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	895	83.85
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	896	101.96
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	897	100.01
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	898	95.46
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	899	79.99
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	900	108.36
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	901	98.69
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	902	80.65
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	903	81.92
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	904	105.79
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	905	120.66
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	906	101.91
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	907	97.4
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	908	106.43
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	909	118.68
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	910	127.77
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	911	120.7
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	912	115.46
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	913	107.73
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	914	112.9
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	915	105.21
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	916	114.18
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	917	96.78
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	918	109.04
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	919	104.62
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	920	116.77
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	921	106.43
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	922	109.68
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	923	109.7
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	924	107.08
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	925	106.44
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	926	135.05
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	927	113.1
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	928	90.34
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	929	105.81
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	930	92.27
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	931	81.94
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	932	78.06

4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	933	85.2
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	934	90.97
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	935	92.96
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	936	129.65
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	937	94.21
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	938	96.12
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	939	112.9
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	940	127.71
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	941	119.97
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	942	118.69
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	943	118.69
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	944	116.1
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	945	140.02
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	946	112.9
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	947	124.49
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	948	94.83
4/17/2009	5	B	DMSO	5	7/23/2009	Kaitlyn/Sam	949	116.13
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	950	113.52
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	951	102.56
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	952	87.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	953	99.33
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	954	85.79
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	955	91
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	956	116.12
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	957	114.17
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	958	114.81
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	959	127.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	960	99.98
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	961	87.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	962	83.85
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	963	66.44
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	964	87.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	965	94.83
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	966	71.59
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	967	82.56
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	968	74.82
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	969	81.29
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	970	67.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	971	67.74
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	972	73.53
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	973	68.38
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	974	97.4
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	975	72.24
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	976	78.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	977	67.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	978	80.63
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	979	74.82
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	980	86.44
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	981	72.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	982	79.98
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	983	72.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	984	78.71
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	985	87.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	986	78.09
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	987	92.23
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	988	96.11
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	989	78.18
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	990	77.41
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	991	83.21
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	992	81.27
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	993	70.31
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	994	93.55
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	995	101.28

4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	996	99.36
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	997	88.97
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	998	85.87
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	999	95.83
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1000	84.5
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1001	78.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1002	92.27
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1003	89.16
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1004	94.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1005	98.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1006	86.97
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1007	92.32
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1008	88.6
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1009	107.1
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1010	99.35
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1011	97.4
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1012	113.52
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1013	102.56
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1014	87.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1015	99.33
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1016	85.79
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1017	91
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1018	116.12
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1019	114.17
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1020	114.81
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1021	127.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1022	99.98
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1023	87.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1024	83.85
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1025	66.44
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1026	87.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1027	94.83
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1028	71.59
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1029	82.56
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1030	74.82
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1031	81.29
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1032	67.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1033	67.74
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1034	73.53
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1035	68.38
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1036	97.4
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1037	72.24
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1038	78.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1039	67.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1040	80.63
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1041	74.82
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1042	86.44
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1043	72.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1044	79.98
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1045	72.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1046	78.71
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1047	87.08
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1048	78.09
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1049	92.23
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1050	96.11
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1051	78.18
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1052	77.41
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1053	83.21
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1054	81.27
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1055	70.31
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1056	93.55
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1057	101.28
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1058	99.36

4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1059	88.97
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1060	85.87
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1061	95.83
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1062	84.5
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1063	78.05
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1064	92.27
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1065	89.16
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1066	94.89
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1067	98.72
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1068	86.97
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1069	92.32
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1070	88.6
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1071	107.1
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1072	99.35
4/17/2009	5	A	32	6	7/16/2009	Mickey/Brad	1073	97.4
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1074	116.75
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1075	96.75
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1076	96.75
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1077	94.82
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1078	92.23
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1079	101.3
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1080	87.72
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1081	113.52
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1082	98.69
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1083	107.08
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1084	93.58
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1085	90.31
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1086	82.56
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1087	81.28
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1088	92.99
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1089	82.56
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1090	92.24
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1091	89.74
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1092	78.05
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1093	68.42
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1094	95.47
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1095	99.46
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1096	82.57
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1097	96.11
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1098	91.59
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1099	64.51
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1100	99.33
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1101	134.82
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1102	74.19
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1103	84.5
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1104	87.76
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1105	92.26
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1106	89.02
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1107	96.78
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1108	95.47
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1109	96.11
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1110	82.57
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1111	100.62
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1112	95.46
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1113	96.11
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1114	97.41
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1115	111.03
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1116	101.27
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1117	103.86
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1118	96.75
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1119	103.85
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1120	85.14
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1121	102.57

4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1122	117.4
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1123	93.53
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1124	93.53
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1125	108.37
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1126	100.62
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1127	99.33
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1128	105.14
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1129	92.24
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1130	81.92
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1131	98.07
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1132	74.89
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1133	83.89
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1134	93.53
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1135	111.59
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1136	102.61
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1137	122.61
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1138	83.89
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1139	102.59
4/17/2009	5	B	32	6	7/23/2009	Kaitlyn/Sam	1140	86.44
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1141	94.17
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1142	72.89
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1143	86.45
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1144	83.29
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1145	87.72
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1146	80.64
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1147	83.86
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1148	77.44
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1149	83.85
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1150	83.21
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1151	81.96
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1152	79.38
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1153	76.76
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1154	78.05
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1155	69.02
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1156	65.87
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1157	68.1
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1158	60.85
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1159	57.42
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1160	63.61
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1161	68.42
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1162	60.8
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1163	69.88
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1164	60.63
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1165	65.16
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1166	74.86
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1167	72.89
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1168	78.76
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1169	74.85
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1170	103.23
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1171	94.18
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1172	80.67
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1173	80.63
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1174	90.3
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1175	93.53
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1176	82.85
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1177	85.84
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1178	77.28
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1179	76.33
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1180	78.58
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1181	77.4
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1182	84.12
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1183	60.63
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1184	83.77

4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1185	69.69
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1186	60.63
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1187	63.24
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1188	69.73
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1189	79.35
4/17/2009	5	A	500	7	7/17/2009	Mickey/Brad	1190	72.99
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1191	101.96
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1192	108.39
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1193	89.68
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1194	120.62
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1195	103.86
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1196	94.17
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1197	112.88
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1198	101.37
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1199	92.27
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1200	110.99
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1201	94.82
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1202	91.09
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1203	92.94
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1204	101.28
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1205	94.82
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1206	94.82
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1207	89.01
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1208	92.9
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1209	74.82
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1210	87.08
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1211	83.21
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1212	96.78
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1213	87.73
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1214	72.89
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1215	69.81
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1216	88.37
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1217	92.26
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1218	64.5
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1219	91.61
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1220	76.77
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1221	103.23
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1222	94.82
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1223	82.56
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1224	102.56
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1225	85.14
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1226	86.52
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1227	96.11
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1228	83.2
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1229	83.87
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1230	103.23
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1231	96.11
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1232	87.72
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1233	103.9
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1234	87.08
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1235	94.21
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1236	123.84
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1237	102.61
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1238	105.85
4/17/2009	5	B	500	7	7/23/2009	Kaitlyn/Sam	1239	93.55
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1240	82.48
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1241	83.9
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1242	80.63
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1243	88.25
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1244	106.62
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1245	82.76
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1246	76.67
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1247	86.85

4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1248	85.79
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1249	86.72
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1250	88.97
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1251	72.42
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1252	81.28
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1253	79.55
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1254	72.34
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1255	80.02
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1256	74.19
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1257	79.34
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1258	71.67
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1259	74.18
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1260	88.76
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1261	69.09
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1262	58.34
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1263	73.37
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1264	76.72
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1265	71.59
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1266	63.88
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1267	72.89
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1268	72.27
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1269	62.62
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1270	73.54
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1271	75.47
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1272	89.66
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1273	61.94
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1274	82.22
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1275	68.31
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1276	96.18
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1277	85.49
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1278	73.76
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1279	92.88
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1280	80
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1281	73.02
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1282	106.52
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1283	79.4
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1284	90.53
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1285	80.94
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1286	97.98
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1287	102.72
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1288	86.27
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1289	81.92
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1290	97.05
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1291	70.96
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1292	98.14
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1293	98.04
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1294	60.48
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1295	83.41
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1296	88.42
4/17/2009	5	A	H ₂ O ₂	8	7/17/2009	Mickey/Brad	1297	95.14
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1298	90.34
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1299	84.5
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1300	88.37
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1301	96.11
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1302	98.04
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1303	89.68
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1304	97.43
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1305	87.72
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1306	104.52
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1307	92.24
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1308	90.31
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1309	105.78
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1310	83.21

4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1311	78.71
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1312	93.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1313	90.95
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1314	98.06
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1315	87.08
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1316	110.95
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1317	80.64
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1318	85.69
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1319	99.98
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1320	69.71
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1321	100.03
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1322	82.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1323	82.57
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1324	79.98
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1325	90.3
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1326	81.96
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1327	41.93
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1328	96.11
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1329	81.96
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1330	76.12
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1331	77.4
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1332	76.76
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1333	58.7
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1334	57.5
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1335	90.41
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1336	40.01
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1337	70.96
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1338	75.49
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1339	85.14
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1340	55.47
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1341	102.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1342	91.7
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1343	49.67
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1344	52.91
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1345	41.93
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1346	94.82
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1347	71.09
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1348	81.92
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1349	77.44
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1350	92.27
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1351	105.14
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1352	87.73
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1353	77.41
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1354	78.05
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1355	100.62
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1356	102.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1357	83.85
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1358	88.37
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1359	87.08
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1360	96.14
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1361	79.34
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1362	79.99
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1363	70.31
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1364	56.12
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1365	81.92
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1366	82.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1367	100.08
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1368	102.56
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1369	88.42
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1370	94.87
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1371	82.58
4/17/2009	5	B	H ₂ O ₂	8	7/23/2009	Kaitlyn/Sam	1372	77.4
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1373	137.64

4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1374	127.72
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1375	142.05
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1376	140.99
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1377	135.11
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1378	134.49
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1379	139.75
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1380	124.28
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1381	145.88
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1382	115.68
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1383	146.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1384	131.61
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1385	140.63
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1386	126.79
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1387	120.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1388	146.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1389	148.36
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1390	130.32
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1391	138.18
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1392	140.73
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1393	127.26
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1394	130.39
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1395	123.33
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1396	113.54
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1397	124.5
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1398	145.81
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1399	141.92
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1400	127.65
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1401	135.46
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1402	134.83
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1403	113.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1404	113.57
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1405	127.39
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1406	123.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1407	120.63
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1408	113.52
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1409	122.66
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1410	110.95
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1411	127.08
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1412	116.28
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1413	123.9
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1414	119.37
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1415	112.88
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1416	120.64
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1417	133.59
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1418	113.53
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1419	117.4
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1420	117.48
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1421	117.42
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1422	109.67
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1423	104.5
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1424	114.89
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1425	139.47
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1426	129.36
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1427	106.5
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1428	99.34
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1429	100.44
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1430	128.41
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1431	126.7
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1432	129.62
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1433	118.17
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1434	138.82
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1435	152.18
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1436	142.96

4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1437	134.31
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1438	118.82
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1439	133.1
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1440	120.68
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1441	146.09
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1442	102.68
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1443	104.05
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1444	127.36
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1445	151.78
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1446	141.61
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1447	134.72
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1448	125.94
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1449	133.54
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1450	106.71
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1451	113.06
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1452	100.83
4/17/2009	6	A	DMSO	9	7/17/2009	Mickey/Brad	1453	94.92
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1454	116.75
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1455	123.33
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1456	101.92
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1457	94.28
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1458	113.54
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1459	105.81
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1460	141.28
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1461	114.17
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1462	109.01
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1463	99.98
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1464	96.77
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1465	96.12
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1466	116.16
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1467	100.03
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1468	112.88
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1469	112.26
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1470	106.02
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1471	107.09
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1472	98.09
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1473	99.43
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1474	128.37
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1475	94.87
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1476	94.82
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1477	97.43
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1478	99.38
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1479	101.47
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1480	103.97
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1481	114.83
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1482	100.63
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1483	101.94
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1484	98.82
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1485	87.1
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1486	114.18
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1487	116.28
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1488	91.67
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1489	90.95
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1490	119.97
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1491	109.04
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1492	106.43
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1493	90.3
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1494	96.14
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1495	116.12
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1496	99.34
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1497	99.34
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1498	98.79
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1499	93.15

4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1500	73.81
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1501	96.77
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1502	94.17
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1503	103.2
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1504	80.65
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1505	101.27
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1506	87.87
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1507	112.26
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1508	87.84
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1509	85.81
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1510	97.81
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1511	95.47
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1512	110.36
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1513	107.72
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1514	96.12
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1515	89.66
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1516	87.72
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1517	99.33
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1518	93.55
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1519	81.12
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1520	104.42
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1521	116.13
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1522	106.47
4/17/2009	6	A	32	10	7/17/2009	Mickey/Brad	1523	92.42
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1524	94.87
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1525	89.66
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1526	76.77
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1527	105.14
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1528	72.27
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1529	97.43
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1530	79.34
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1531	85.78
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1532	90.95
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1533	73.56
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1534	78.05
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1535	89.66
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1536	72.24
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1537	60
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1538	95.46
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1539	90.36
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1540	67.08
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1541	87.08
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1542	87.08
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1543	91.65
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1544	108.41
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1545	74.86
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1546	70.45
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1547	101.62
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1548	102.9
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1549	102.63
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1550	89.89
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1551	89.84
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1552	97.01
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1553	84.79
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1554	87.74
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1555	86.72
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1556	88.42
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1557	104.51
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1558	99.41
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1559	96.18
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1560	96.37
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1561	80.07
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1562	99.34

4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1563	93.1
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1564	121.23
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1565	92.24
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1566	88.6
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1567	86.67
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1568	72.89
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1569	78.05
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1570	91.06
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1571	95.51
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1572	92.92
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1573	94.24
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1574	107.14
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1575	82.12
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1576	78.14
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1577	121
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1578	111.03
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1579	96.75
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1580	107.72
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1581	99.46
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1582	100.67
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1583	112.19
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1584	112.99
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1585	125.19
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1586	103.25
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1587	109.67
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1588	104.51
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1589	110.45
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1590	99.35
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1591	89.84
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1592	113.41
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1593	109.72
4/17/2009	6	B	32	10	7/24/2009	Kaitlyn/Sam	1594	111.59
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1595	132.87
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1596	111.29
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1597	112.26
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1598	146.05
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1599	122.55
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1600	103.85
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1601	117.42
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1602	145.13
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1603	122.61
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1604	108.39
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1605	114.99
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1606	81.27
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1607	101.27
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1608	114.25
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1609	117.5
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1610	113.61
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1611	112.35
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1612	101.3
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1613	101.37
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1614	90.34
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1615	99.54
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1616	101.3
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1617	108.21
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1618	119.99
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1619	93.53
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1620	126.42
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1621	89.77
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1622	90.95
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1623	90.32
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1624	89.66
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1625	89.07

4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1626	88.45
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1627	95.47
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1628	114.84
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1629	85.38
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1630	97.76
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1631	83.21
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1632	92.9
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1633	82.22
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1634	84.56
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1635	94.19
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1636	100.62
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1637	93.2
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1638	101.92
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1639	116.13
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1640	116.75
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1641	110.57
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1642	81.96
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1643	102.68
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1644	98.14
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1645	91.74
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1646	103.3
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1647	101.91
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1648	83.56
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1649	98.74
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1650	105.94
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1651	107.7
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1652	76.82
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1653	94.82
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1654	78.79
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1655	75.93
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1656	108.09
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1657	116.76
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1658	120.18
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1659	87.36
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1660	107.72
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1661	91.6
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1662	105.85
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1663	109.02
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1664	107.99
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1665	110.45
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1666	105.79
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1667	92.96
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1668	91.6
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1669	105.85
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1670	109.02
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1671	107.99
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1672	110.45
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1673	105.79
4/17/2009	6	A	500	11	7/17/2009	Mickey/Brad	1674	92.96
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1675	99.33
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1676	108.37
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1677	92.24
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1678	119.33
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1679	96.11
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1680	98.69
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1681	79.36
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1682	84.5
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1683	95.46
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1684	90.95
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1685	98.05
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1686	136.74
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1687	87.11
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1688	78.71

4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1689	87.76
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1690	83.23
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1691	87.72
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1692	92.23
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1693	66.45
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1694	96.75
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1695	96.11
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1696	96.75
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1697	92.24
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1698	99.34
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1699	84.5
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1700	115.46
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1701	94.82
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1702	104.49
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1703	88.37
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1704	82.57
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1705	90.97
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1706	92.89
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1707	83.85
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1708	115.46
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1709	96.86
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1710	85.76
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1711	103.85
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1712	89.68
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1713	98.69
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1714	79.43
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1715	99.79
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1716	82.81
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1717	82.57
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1718	90.58
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1719	81.33
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1720	94.89
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1721	85.49
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1722	78.06
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1723	80.91
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1724	90.3
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1725	109.38
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1726	101.93
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1727	90.95
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1728	90.95
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1729	85.34
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1730	83.91
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1731	81.92
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1732	79.21
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1733	77.91
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1734	87.81
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1735	85.34
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1736	83.91
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1737	81.92
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1738	79.21
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1739	77.91
4/17/2009	6	B	500	11	7/24/2009	Kaitlyn/Sam	1740	87.81
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1741	138.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1742	125.78
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1743	157.49
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1744	150.29
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1745	138.13
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1746	129.01
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1747	130.3
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1748	136.89
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1749	147.71
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1750	115.46
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1751	127.11

4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1752	113.14
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1753	126.5
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1754	126.5
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1755	121.92
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1756	153.53
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1757	162.63
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1758	118.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1759	132.3
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1760	121.91
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1761	126.62
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1762	163.1
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1763	124.22
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1764	143.21
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1765	140.04
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1766	171.72
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1767	150.09
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1768	118.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1769	117.05
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1770	139.97
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1771	123.84
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1772	137.13
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1773	137.99
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1774	115.67
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1775	132.95
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1776	122.29
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1777	99.43
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1778	110.45
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1779	136.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1780	118.04
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1781	148.46
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1782	119.34
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1783	108.15
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1784	121.91
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1785	145.18
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1786	118.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1787	124.55
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1788	141.79
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1789	144.55
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1790	131.71
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1791	149.45
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1792	129.01
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1793	102.56
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1794	113.53
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1795	118.04
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1796	111.06
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1797	130.29
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1798	125.13
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1799	116.68
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1800	124.04
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1801	119.33
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1802	130.35
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1803	142.75
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1804	127.84
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1805	113.53
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1806	133.91
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1807	156.77
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1808	104.52
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1809	145.91
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1810	87.1
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1811	106.86
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1812	145.94
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1813	107.07
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1814	136.11

4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1815	105.14
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1816	116.96
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1817	108.55
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1818	76.8
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1819	129.92
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1820	121.95
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1821	159.45
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1822	149.27
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1823	138.18
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1824	147.17
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1825	169.76
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1826	135.53
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1827	152.91
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1828	142.55
4/17/2009	6	A	H ₂ O ₂	12	7/17/2009	Mickey/Brad	1829	166.42

Appendix E: Fall 2008 Live Animal Study

During the Fall of 2008 our team undertook a live animal study investigating the effects of 17α -ethynylestradiol (EE₂) on the reproductive efficacy of fathead minnows (*Pimephales promelas*). The two endpoints used to measure reproductive efficacy in this study were sperm motility and genotoxic damage to sperm cells. This experiment was unsuccessful due to a variety of challenges. Though the experiment did not provide information on the intended endpoints the lessons it provided did much to inform the approaches and experimental design of our main experiment. The following material should be understood as a prologue to our main experiment and helps to better elucidate the rationale behind many of our decisions.

Proposed Methodology for Fall 2008 Live Animal Study

The following section details the intended methodology for the Fall 2008 live animal study. This methodology was written before our experiment took place. Because of a series of challenges, we were unable to follow through with this methodology. However, it still provides a clear picture of the intended design of our first experiment.

Introduction

For the purpose of completing the research within the limited time and budget available, the model system for the study will be the fathead minnow, a species of fish that has been previously used in many experiments (Ankley et al., 2002; Ankley, 2004; Martinovic et al., 2008).

As explained previously, fathead minnows are an ideal fish to study given the constraints placed on this study. They are relatively cheap and easy to raise. Also, they are small in size compared to other model fish, allowing for a larger sample size in limited lab space. Overall, because the endocrine system is highly conserved among vertebrates, the endocrine responses of fathead minnows are representative of other vertebrate organisms including humans.

Vitellogenin production in male fish is an indication that the fish have been exposed to and are responding to endocrine disrupting compounds. An accepted means of inducing VTG production in male fish is by injecting estrogenic compounds such as 17 β -estradiol (E₂) or 17 α -ethynylestradiol (EE₂). EE₂ is useful in an experiment measuring the effects of EDCs. As a synthetic estrogen, it is designed to bind to estrogen receptors and can be expected to have disruptive effects similar to other EDCs that mimic estrogen (Ankley et al., 2002).

Finally, the group assumes that sperm motility (as determined by sperm velocity, movement duration and directionality), sperm concentration (defined as the amount of sperm cells in a given volume of semen), and sperm genetic quality (measured as the

extent of DNA damage) are valid measures of reproductive efficacy. These assumptions are based upon the facts that slight decreases in sperm motility, quantity, and quality could negatively affect fertilization.

Husbandry and Materials

Upon approval from the Animal Care and Use Committee, all fathead minnows will be purchased from Aquatic Research Organisms, Inc., a reliable supplier of invertebrates and fish that include fathead minnows, for use in research. For each male purchased, two females will be acquired and placed into a tank with an individual male to encourage spermiation. Of the forty-eight males purchased, thirty-six will be used in the experiment. A random sample of six will be initially sacrificed and bled to determine if there is any vitellogenesis in males *prior* to the experiment through the use of a vitellogenin (VTG) immunoassay. This will allow us to assure our fish population has not been affected by EDCs before our experiment. The remaining six male fish are extras to allow for possible morbidity or mortality that may occur due to handling associated with shipment and acclimation to the new system at the University of Maryland. Seventy-eight female fish will be obtained as well, only seventy-two of which are intended for experimental use (the extras, again, are in the case of morbidity or mortality associated with handling and acclimation).

Fish will be kept in 2.8 L aquaria in an Aquatic Habitats™ stand-alone fish keeping system that utilizes a water recirculation system. Forty-eight of these 2.8 L tanks will be obtained through a reliable retailer and used throughout the experiment. The water

will be dechlorinated using sodium thiosulfite and essential electrolytes will be provided by adding 1 mg/L of Instant Ocean[®] to the culture system's water. Water temperature will be held at room temperature (25°C) as fatheads are routinely spawned at room temperature. (Speirs, 2000) To further facilitate spawning, a longitudinally-cut PVC pipe will be placed in each tank, to serve as a spawning substrate. Procedures will be established that provide for regular cleaning and water quality analysis of the fish keeping system weekly. The minnows will be exposed daily to a strict photoperiod including 16 hours of light and 8 hours of dark. They will be offered a commercial feed *ad libitum* twice each day. Once again, all fish will be cared for according to an approved Animal Care and Use Committee protocol. (Liney, 2006)

Experimental Procedure

The thirty-six male fish will be housed in individual 2.8 L tanks and divided into six equal experimental groups, one of which will be the control. Additionally, seventy-two females will be placed on the basis of two females per tank to induce spermiation in each male minnow throughout the course of the experiment. Each group of male fish will be intraperitoneally (*i.p.*) injected with equivalent volumes of physiological ethanol solution containing various diluted concentrations of EE₂. The six treatment concentrations will be 0, 0.01, 0.1, 1, 10 and 100 mg of EE₂ per kg of body weight, which represents an experimental range that approximates concentrations of estrogen injected into fish species in other published studies (Kashiwada et al., 2007; Tilton & Schlenk,

2001). Prior to the initial injection, the wet weight of each male will be determined after sedating the minnows in an anesthetic bath (70 mg/L buffered MS-222).

The injections will be handled the same way across each treatment. Each group of fish will be injected in a staggered fashion every seven days at the beginning of a 4-week period for a total of four injections. The staggered method will allow for greater feasibility in the post-data collection analysis of each experimental group of fish. The weeklong wait after each injection will better ensure adequate EE₂ exposure to and circulation through the minnow's endocrine system. After each injection, the fish will be placed in separate 2.8 L tanks isolated from the stand alone fish keeping system for 15 minutes to monitor the injection site for potential bleeding and leakage and ensure the fish's recovery from the anesthesia before being returned to its experimental tank.

Data and Analysis

After the staggered four weeks of EE₂ injections, males from each group will be euthanized and tissues collected for analyses of quantifiable data. The experiment is designed to determine whether a positive correlation exists between the feminization of male fish (as measured by VTG levels in the blood plasma) and reproductive efficacy (as measured by sperm motility, quantity, and quality). Therefore, quantitative data will be collected in the form of the amount of VTG produced in the male minnows and via several experimental methods measuring reproductive viability, including the velocity and duration of sperm movement, sperm density and volume, and sperm and gonadal cell DNA integrity.

The first set of data obtained will be the blood samples collected from all the male fish in each experimental group through a caudal tail clip, (Nash et al., 2004) which will require sacrificing the fish. The blood will be collected in thirty-six separate vials and immediately heparin, or another appropriate anticoagulant, will be added to each sample. Subsequently, the blood samples will be stored in a freezer and shipped to a supporting laboratory where VTG enzyme-linked immuosorbent assay (ELISA) will be conducted on the blood plasma. The immunoassay will be used to measure the amount of VTG produced in each male fish from all of the experimental groups of minnows, and to confirm that VTG production is dose-dependent on estradiol injections, as found by earlier experiments (Pollack et al., 2003).

Following the collection of blood samples, both lobes of the testes from all the experimental male fish will be excised and then the sperm from each lobe will be removed. Excision of the testes, as opposed to expressing the release of sperm by squeezing the fish, eliminates any inaccuracies due to urine and water contamination. Both lobes of the testes will be weighed after the sperm has been removed from them, to determine whether EDC exposure has an effect on the size and development of the testes. The sperm samples will be transferred to the laboratory for analysis and divided into three portions, divided appropriately by the amount required for the assessment of motility, quantity, and quality. Sperm motility will be determined through the use of fluorescent stain-based computer-assisted sperm analysis (CASA) software that quantitatively analyzes sperm motility (Nash et al., 2004). The CASA calibration settings will be set appropriately according to the specific shape and characteristics of the sperm of fathead minnows. Additionally, using a hemacytometer, sperm concentrations will be

determined in terms of the amount of spermatozoa (packed cell volume/total semen volume) produced by each fish. Sperm quality, defined as the level of DNA damage (in terms of strand breaks and alkali labile sites) will be measured using the comet assay. Additionally, the comet assay will be applied to gonadal tissue for one or both lobes of the testes to analyze the environment in which the sperm is produced. As previously mentioned, the comet assay has been successfully used in past studies as an analytical tool to study DNA integrity in blood and gill cells of fish and in human sperm (Morris et al., 2002).

The collected data will be analyzed through the creation of several graphs that display the correlation between vitellogenin production and the three aforementioned tenets of sperm efficacy (motility, quantity, and quality). The first graph will plot vitellogenin levels of each experimental group against the amount of EE₂ injected. A positive correlation will replicate results found in the literature and will reaffirm that the estradiol injections have had feminizing effects on the male fish in a dose-dependent manner. The second graph will plot sperm motility against the concentration of EE₂ injected *i.p.* (in mg/kg) which will show what effect increasing concentrations of estradiol have on sperm motility. Sperm concentration will also be graphed against EE₂ concentration levels. This graph will indicate the relationship between endocrine disruption via estradiol and the resulting spermatozoa. A fourth graph will plot DNA damage in the sperm against the EE₂ concentrations for each group, which will demonstrate how increases in the estradiol levels affect this specific tenet of reproductive capability in sperm. The fifth and final graph will plot DNA damage in gonadal cells versus the estradiol dosages, adding to the knowledge of the effect of various

concentrations of EE₂ on reproductive viability. Even in the case that the experiment does not reveal dose-dependent VTG production, the graphs referenced above will be generated, as the varying amounts of EE₂ may still have a quantifiable and comparable effect on the reproductive efficacy of male fathead minnows.

Results and Their Context

Procuring data on VTG production levels and sperm efficacy will help explain the effects of EDCs and feminization on fish reproductive viability. The presence of vitellogenin in male fish is an accepted indication that the fish have been stimulated by an estrogenic compound to produce a protein normally only produced in the female liver. However, a dose-dependent correlation between EDC exposure and VTG levels is not necessary for analysis of data, as EDC exposure may still affect reproductive viability without inducing vitellogenesis.

The research is likely to yield one of two main conclusions. The data may support the null hypothesis that there is no correlation between varying concentrations of EDC injected and sperm efficacy, suggesting that endocrine disruption has no effect on male fish reproductive capability. Conversely, the data may reveal a correlation between EDC exposure and one or more tenets of sperm efficacy, suggesting that feminization does affect reproductive ability. With either conclusion, information will be added to the scientific community regarding the effect of EDCs on fish populations and reproductive success in organisms.

Beyond the tests performed in this study, follow-up experiments are needed. Especially in the case that an EDC induced effect is found on sperm viability, further research will be needed to determine the mechanism behind the phenomenon. In addition to examining sperm viability, testing the fertilization rate of eggs by sperm procured from vitellogenin-producing males would be necessary to conclusively determine that reproductive success, in terms of steady populations of future generations of fish, is affected by EDCs.

Benefits and Limitations

The experiment will shed light on the relevance of previous studies of EDCs and feminization to the health of fish populations by clarifying the connection between endocrine disruption and reproductive efficacy. Additionally, the particulars of our experiment hold a certain advantage as well. Because of the use of various concentrations of EE₂, a standard curve can be created to correlate EDC exposure to the three factors defining sperm viability, allowing the scientific community to possibly gauge the effects of different amounts of EE₂ on reproductive efficacy in fish.

The experiment does have certain drawbacks. The use of live animals in this study could lead to unforeseen delays. During the process of the laboratory experiment, there may be mortalities within the fish population, for example as a result of disease. Moreover, the complexity of the experiment leads to a high material cost to cover tank systems, lab space, assay kits, and other scientific paraphernalia. One limitation to the study is that it does not directly measure fertilization rates, so it must make the assumption that sperm motility, concentration, and integrity are reasonable indicators of the male's ability to fertilize. Additionally, the conditions present in the breeding tanks might not reflect the natural environment of the fathead minnows, causing changes in behavior and breeding patterns.

Challenges with the Fall 2008 Live Animal Experiment

A variety of issues prevented the successful completion of our study. Mortality and morbidity in our experimental animal population was the primary cause of problems. Unbeknownst to us, the fish that we received were already in poor health. We received fish with blood flukes and trematodes, weakening their ability to cope with stress and adding unknown variables into the experiment. A large number of fish died before any injections took place. The supplier provided us with additional fish but unfortunately these fish were of a much smaller size than the first shipment.

The size of the fish proved a problem in two respects. Most importantly, the smaller fish provided smaller testes, making excising and examining the testes extremely difficult. This reduced the number and quality of sperm samples we were able to obtain. Even when the testes could be excised the amount of sperm produced was limited. In addition the smaller fish were less robust and able to withstand the injection regimen. Mortality was high due to the stress of anesthetizing and injecting the small fish.

Following the high mortality after the first round of injections, a simple experiment was performed to see if the fish were suffering from ethanol toxicity. The experiment indicated ethanol may have been responsible for some of the mortality so corn oil was used to dissolve the EE₂ injections. Though survivorship improved after the switch mortality was still high enough to affect our results.

Due to the high mortality rates, we also encountered several problems in the data analysis. Entire experimental groups of fish died, we were unable to effectively analyze our data. In addition the replicates for each set were drastically reduced from our original number of n=6. With numbers of n=0, 1, or 2 for many groups, the error bars for the data we were able to collect

were so large as to render the data almost entirely meaningless. It was therefore impossible to draw any conclusions about the effect of EE₂ injections on reproductive efficacy.

Data from the Fall 2008 Live Animal Study

Several problems occurred during the experiment and that resulted in very few successful dissections of the male fathead minnows, collections of blood plasma, and extractions of sperm. After the initial high mortality period our experiment was scaled back to use a block design with 24 fish split into two blocks. Of the 12 fish in block one, only 6 lived until the date of sacrifice, and only 5 of those had extractable testes. Of the 12 fish in block two, only three fish survived and none of these was a control. Another notable issue was that all of the control fish in block 2 had died before they could be euthanized and dissected.

CASA

- Fish E – 1.0 mg of EE₂ per kg of body weight

Note: There were problems with performing the assay on this fish.

<i>Chamber</i>	<i>Concentration (M/ml)</i>	<i>Percent Motile (%)</i>	<i>Track Speed</i>
1	69.4	38	71.51
2	76.1	11	49.9
4	102.2	0	19.9

- Fish F – 0.1 mg of EE₂ per kg of body weight

<i>Chamber</i>	<i>Concentration (M/ml)</i>	<i>Percent Motile (%)</i>	<i>Track Speed</i>
1	90.1	18	50.9
2	90.1	9	44.0
4	76.2	3	38.2

- Fish G – 10.0 mg of EE₂ per kg of body weight

<i>Chamber</i>	<i>Concentration (M/ml)</i>	<i>Percent Motile (%)</i>	<i>Track Speed</i>
1	33.9	50	51.9
2	26.5	17	42.8
3	3.9	12	41.9
4	20.1	3	35.9

- Fish H – 0.0 mg of EE₂ per kg of body weight (Control Fish)

<i>Chamber</i>	<i>Concentration (M/ml)</i>	<i>Percent Motile (%)</i>	<i>Track Speed</i>
1	3.9	9	52.5
2	2.2	7	41.1
3	0.5	8	38.8
4	1.7	7	30.4

- Fish L – 0.0 mg of EE₂ per kg of body weight (Control Fish)

<i>Chamber</i>	<i>Concentration (M/ml)</i>	<i>Percent Motile (%)</i>	<i>Track Speed</i>
1	6.5	46	49.4

Comet Assay

- Block 1

<i>Fish</i>	<i>Concentration (mg of EE₂/kg of body weight)</i>	<i>Number of Cells</i>	<i>Length (μm)</i>	<i>Standard Deviation</i>
E	100	100	57.1221	13.46247
F	0.1	104	45.68635	10.58672
G – CONTROL	0.0	100	50.2445	14.54189
H	0.1	62	68.15161	9.92666

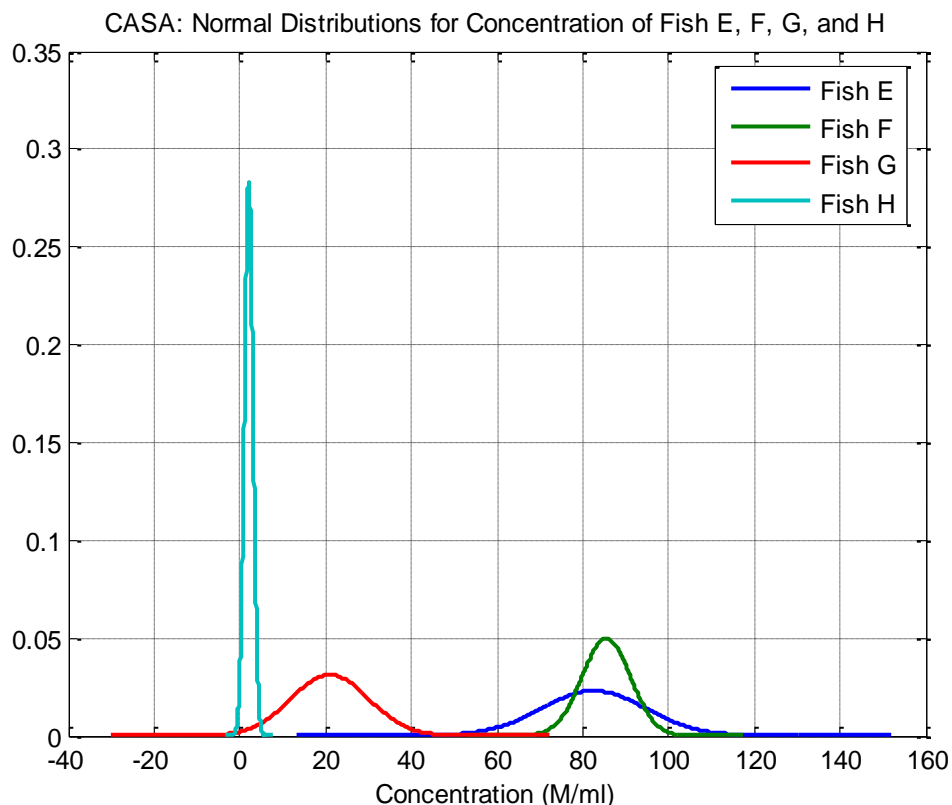
- Block 2

<i>Fish</i>	<i>Concentration (mg of EE₂/kg of body weight)</i>	<i>Number of Cells</i>	<i>Length (μm)</i>	<i>Standard Deviation</i>
Q	0.1	70	106.4919	33.36644
U	100	29	109.9818	20.75335
V	0.1	179	72.09374	25.59331

Data Analysis

CASA

<i>Fish</i>	<i>Concencen. Average (M/ml)</i>	<i>Concencen. Standard Deviation</i>	<i>Concencen. Variance</i>	<i>Percent Motile Average (%)</i>	<i>Percent Motile Standard Deviation</i>	<i>Percent Motile Variance</i>	<i>Track Speed Average</i>	<i>Track Speed Standard Deviation</i>	<i>Track Speed Variance</i>
E 1.0	82.57	17.33	300.3233333	16.33	19.55	382.33	47.10	25.92	671.76
F 0.1	85.47	8.03	64.40	10.00	7.55	57.00	44.37	6.36	40.42
G 10.0	21.10	12.78	163.28	20.50	20.50	420.33	43.12	6.60	43.60
H 0.0	2.08	1.41	1.99	7.75	0.96	0.92	40.70	9.11	83.03
L 0.0	6.50	N/A	N/A	46.00	N/A	N/A	49.40	N/A	N/A



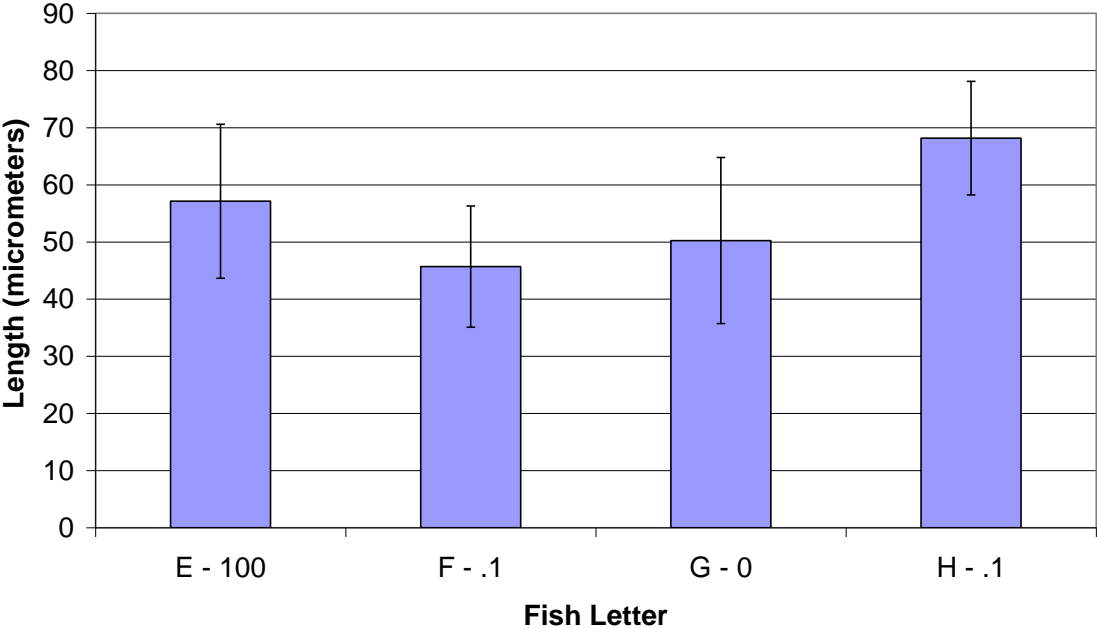
Due to the fluctuations and unsteadiness of the averages, standard deviations, and high variances associated with each fish, it would be reasonable to assume that there is no statistically-provable correlation to be found between EE₂ concentration and sperm concentration, motility, and speed with this given set of data. The variances of each fish in each category effectively eliminate most statistical tests from being relevant to the data because basic assumptions are not met. Moreover, the discrepancy between the two control fishes is even more alarming and makes the data less trustworthy.

Comet Assay

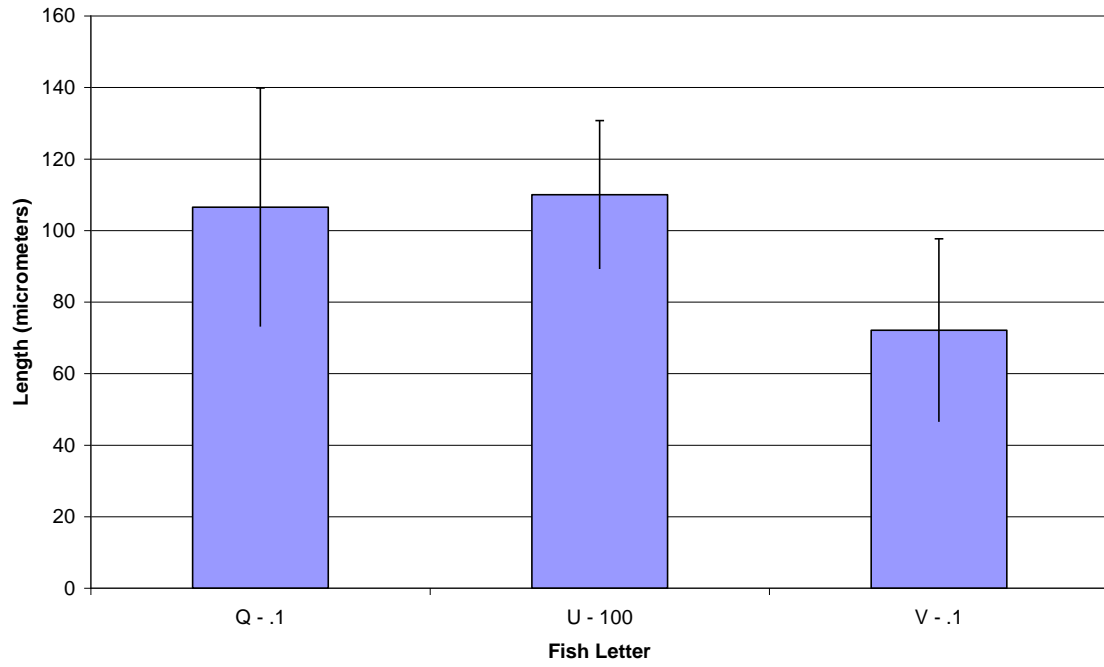
Since there are no controls in the second block, confounding variables cannot be accounted for in any kind of statistical analysis and so none should be performed. With the first block, the control fish allows for some analysis.

<i>Fish</i>	<i>Difference in Length from the Control (FISH - G)</i>
E (100)	6.8776
F (0.1)	-4.55815
H (0.1)	17.90711

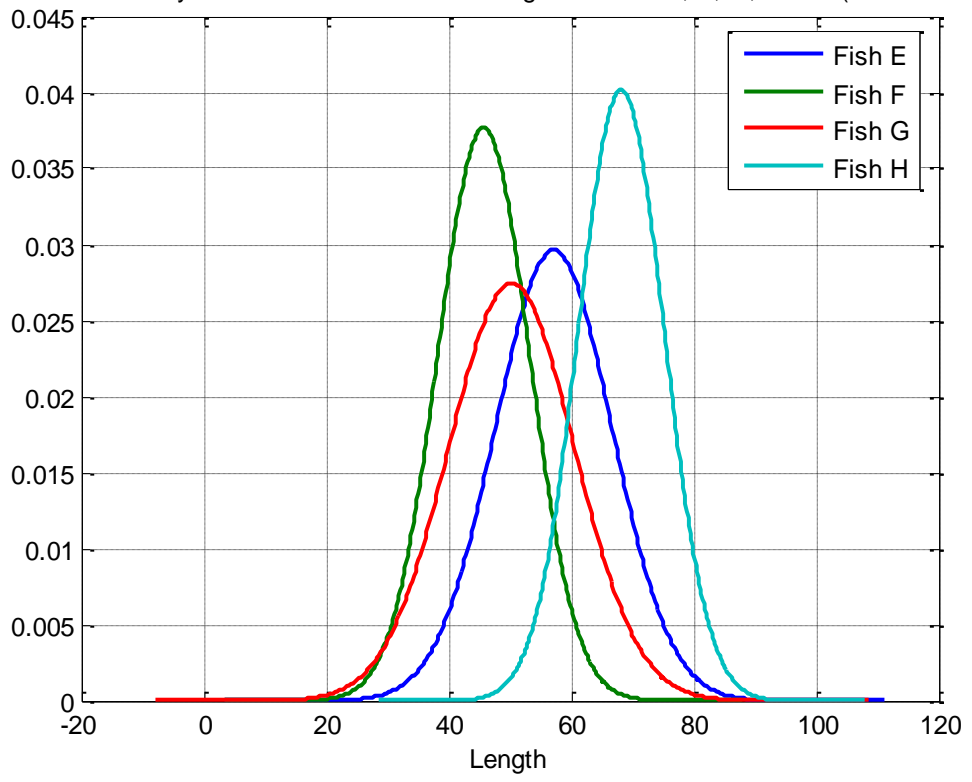
Block 1: Length of Comet Tails



Block 2: Length of Comet Tails



Comet Assay: Normal Distributions for Lengths of Fish E, F, G, and H (First Block)



The fact that these distributions are very similar is misleading. In the first table, where fish G's length is subtracted from the other fishes' lengths, the difference between the result with fish F and fish H is significant because they have the same concentration. This alone makes analysis very hard because, with the confounding variables accounted for, the assumption requirements of many statistical tests cannot be met. Also, looking at the two sets of bar graphs, the fact that a correlation cannot be established is easy to recognize.

Live Animal Data Conclusions

Due to the cost in time and resources the ELISA assay and full statistical analysis were not performed. Rather, it was agreed that our efforts would be better spent in applying the lessons from our first experiment to the design of a new experiment.

Options that We Considered For Spring 2009

Though the final design of our second study was discussed at length in the thesis, this excerpt from our initial proposal provides a list of the alternatives we considered and may be helpful in providing context for our main experiment.

In Vivo Study

It will be unfeasible for us to conduct another controlled live animal experiment this semester. At one point, we revisited an idea that we had considered very early on: an in vivo study. Such a study would involve catching fish from the Potomac and running assays, such as the comet assay on these wild fish. However, there are too many practical obstacles to such an experimental design. It would be difficult to establish a baseline for correlations between

different experimental groups. All of the fish we catch would be from the same supposedly dirty environment and we would not be able to compare any data we collected to “clean” fish data. There are also the legal and administrative obstacles of acquiring the necessary permits from the Maryland Department of Natural Resources. We also would need to find an electro-shocker or other equipment and we probably would not have enough time to collect enough fish to get significant data.

Cell-line

It is much more reasonable to run an *in vitro* study instead of an *in vivo* study. With this in mind, we considered purchasing a gonadal cell line. There is a cell line of rainbow trout gonadal cells available called RTG-2. We could expose the cell line to different amounts of estrogen and measure genotoxicity levels of the cells. Ultimately, we would have to change our research question and focus. The RTG-2 cell line includes both male and female gonadal cells but our research has all focused exclusively on reproductive efficacy in males. We would also need access to a hood because cell lines need sterile environments. This option is still viable if we can find lab space and tweak our research question.

Our Proposed Spring 2009 Experiment (Direct Exposure of Sperm to EE₂)

Our unsuccessful experiment with a fish model system has led us to contemplate possible *in vitro* components to answer our research question. Theoretically, exposure of our fathead minnows with injections would have internally saturated the fishes’ body with 17-β ethynylestradiol (EE₂). EE₂, a very potent estradiol, would have bound to estrogen receptors found throughout the body, such as in the brain, liver, and gonads. There are several pathways by which EE₂ injections could have affected the sperm cells of our male fish. First, EE₂ could have bound to hormone receptors in the brain, thereby triggering a cascade of hormonal responses

throughout the body. The normalcy of the hypothalamic-pituitary-gonadal axis would have been compromised leading to a myriad of abnormal responses. Second, as the most pertinent part of this axis, the gonads could have been inundated with potent EE₂, and the new sperm cells created through spermatogenesis would have been affected. Third, EE₂ could have directly entered the gonads and come into direct contact with sperm cells. Our future experiments will focus on elucidating the latter.

We plan on directly exposing fresh and frozen fish sperm samples to EE₂. By using variable durations and concentrations, we would like to see what effect EE₂ has on the quality of the sperm. Sperm quality will be measured in a number of ways: genotoxicity, apoptotic ratio, and cytotoxicity. The comet assay will be used to assess the quantity of DNA strand breaks and possibly the number of apoptotic cells. We are currently looking into possible cytotoxicity stains, such as neutral red, trypan blue, and the MTT assay. Neutral red and trypan blue are both vital stains which indicate whether or not a cell is alive. Neutral red will be taken into a viable living cell through normal cellular transport mechanisms, staining the cell red. If a cell has died, neutral red cannot traverse the cellular membrane and therefore the cell will remain un-dyed. In contrast, trypan blue cannot cross the cellular membrane of a living cell but can cross that of a dead cell, coloring the cell blue. The MTT assay is a colorimetric assay based on the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan by mitochondrial reductase. The conversion of MTT to purple formazan would occur in viable cells but not in cells with compromised mitochondrial activity which is often associated with an unhealthy cell. Due to the significant amount of error from our past experiment, we plan on performing multiple types of sperm quality assessments.

We are also planning on testing variables beyond simply the duration and concentration of EE₂ exposure. Due to the lipophilic nature of EE₂, the hormone receptor is found in the interior of the cell. Like other hormones, EE₂ would pass through the membrane to bind to the receptor, activating a cascade of cellular processes. So, one effect of EE₂ on sperm cells would be binding

to these receptors and activating these pathways. However, excess EE₂ could also accumulate in the cytoplasm of the cell and cause chemical changes separate from binding to its receptor. Therefore, we are considering using tamoxifen, an estrogen receptor inhibitor, to isolate the cellular changes caused by EE₂ binding and other cytoplasm chemistry.

Another possible variable are natural EE₂ metabolites. One of the major components of EE₂ breakdown is performed in the liver by Cytochrome p450 enzymes. Liver chemical degradation of EE₂ could impact the fish in two ways. First, active EE₂ concentrations would decrease and the overall effect on the fish would be dampened. Second, accumulation of EE₂ metabolites could produce a novel effect. Therefore, we are considering using liver extract to treat concentrations of EE₂ before exposure to sperm cells to stimulate realistic degradation of EE₂ in fish.

Glossary

androgen: any steroid hormone that helps regulate the development of male features, such as male sex organs and secondary sex characteristics

aneuploidy: possessing an abnormal number of chromosomes

aromatase: the enzyme responsible for the production of estrogens from androgens

comet assay: assay using an electrophoresis chamber to determine levels of genetic damage

Computer-Assisted Sperm Analysis (CASA): software which digitizes images of groups of sperm and quantifies the speed and direction of individual sperm

cryopreservation: process where cells or tissue is preserved by exposure to sub-zero temperatures

cytotoxicity: the degree to which a chemical is toxic to a cell

dimethylsulfoxide (DMSO): a commonly used solvent which easily permeates cell membranes

Electrophoresis: the individual movement of particles when exposed to a uniform electric field

Endocrine Disrupting Compounds (EDCs): chemicals that interfere with the endocrine system of organism by altering homeostatic hormonal balances, resulting in various physiological impacts, especially to the reproductive system.

endocrine system: a complex signaling network that releases hormones through the bloodstream to regulate the functions of bodily tissues and organs; highly conserved across vertebrates; consists of various glands including the gonads and the thyroid and pituitary glands.

estradiol (E₂): an estrogen produced in the ovaries of female animals

estriol (E₃): an estrogen produced in the ovaries of female animals

estrogen: a group of steroid compounds functioning as the primary female sex hormone

estrone (E₁): an estrogen produced in the ovaries of female animals

ethynylestradiol/ 17- α -ethynyl estradiol/EE₂: a synthetic estrogen found in many birth control treatments that is twice as potent as any naturally-occurring form of estrogen

fish kills: a localized occurrence of abnormally high mortality in fish populations, such as occurred in the Shenandoah and Potomac River systems in 2002; generally considered to be a result of stress from environmental or anthropogenic sources.

flow cytometry: a technique used to count and differentiate microscopic particles (like cells) based on a pre-set parameter (size, fluorescence, etc.)

Fluorescence-Activated Cell Sorter (FACS): specialized flow cytometry machine

genotoxicity: level of damage present in genetic material

gonads: the sexual organs in males and females responsible for gametogenesis and sex hormone production; termed the ovaries (in females) and the testes (in males)

hepcidin: an important iron-regulating hormone in animals that also has anti-microbial qualities

hermaphrodite: an animal that possesses both functional male and functional female reproductive organs

histology: study of cells and cell tissue on a microscopic level

hypothalamic-pituitary-gonadal (HPG) axis: a grouping of the hypothalamus, pituitary and gonadal glands, which combine to help develop and regulate many of the body's internal functions, such as the reproductive and immune systems

intersex: a condition where an organism of one sex possesses sexual tissue of the opposite sex; distinct from hermaphroditism; may also result in incomplete or mixed secondary sexual characteristics, or behavior changes that restrict or block courtship and mating

masculinization: the biological development of sexually differentiated male characteristics, caused by androgenic hormonal effects

oocytes: immature female egg cells

ova-testes: sexual organs that are primarily male, with some female attributes

Propidium Iodide staining: a staining technique used to differentiate dead or dying cells

receptor: a protein molecule, residing in the plasma membrane or cytoplasm of a cell, to which a ligand binds and enters the cell or transmits a signaling cascade

reproductive efficacy: for the purposes of this study, defined as the survival rate and genetic integrity of the control and experimental spermatazoa

sequential hermaphrodite: organism that can change sex, from either male to female, termed protandry, or from female to male, called protogyny

simultaneous hermaphrodite: organism that contains both male and female sex organs at the same time; sometimes reproduce asexually

spermiation: the time at which males begin producing large quantities of sperm

SYBR-14: a stain that only colors living cells

testes: the reproductive gland in a male vertebrate, which are the source of spermatozoa and the androgens, and normally occur in pair

vitellogenin: an egg precursor protein naturally found only in females; presence in males indicates disruption of the endocrine system

wastewater effluent: flow into a river, stream, lake, or other waterway of sewage, fertilizers in solution, or liquid industrial waste

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