Gait Following Transcutaneous Osseointegrated Limb Replacement in Swine; A Pilot Study

By

Alyson Adelle Boyer

Submitted to the graduate degree program in BioEngineering and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Chairperson Terence McIff

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Carl Luchies

_____

Sara Wilson

Date Defended: May 29, 2014

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The Thesis Committee for Alyson Adelle Boyer

certifies that this is the approved version of the following thesis:

Gait Following Transcutaneous Osseointegrated Limb Replacement in Swine; A Pilot Study

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Chairperson Terence McIff

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ABSTRACT

It is currently estimated that one in 142 people in the US are living with an amputation. For lower-limb amputees, the socket prosthetic presents a number of clinically-recognized issues at the socket-to-stump interface, including blisters, sores, swelling, and heterotopic ossification. A novel alternative to the socket has been developed and implemented in over 250 patients in Europe alone. This new approach involves an implant anchored in bone (osseointegrated) that passes through the skin (transcutaneous) and attaches to an external prosthetic, eliminating the socket and the problems that accompany it. This innovative solution has not yet been approved in the US due to a lack of human clinical trials, a high infection rate in Europe, and the lack of a permanent seal at the skin-to-implant interface. To further investigate infection and wound healing at the stoma site, an animal model must be developed. This pilot study is also the first step to obtaining FDA approval in the US.

A hind leg amputation was performed by an orthopedic surgeon on four adult Yucatan Mini Pigs. The length of the study varied for each animal: 5, 8, 10, and 11 weeks post-surgery. Specific aim I was to develop and validate a surgical procedure and perform a trans-tibial hind leg amputation, during which a titanium implant was inserted through the skin into the tibial canal. A prosthetic was attached at 2 days post-op.

The objective of specific aim II was to promote wound healing while preventing infection. The wound was cleaned and the dressings were changed every 2-4 days. Two methods of wound cleaning were investigated: H_2O_2+H_2O solution vs. soap+H_2O solution. Bacterial swabs were collected at each dressing change to monitor the wound for infection.

Infection was successfully prevented in all animals; soap+H_2O was proven to be a sufficient method for cleaning the wound and preventing infection.

The objective of specific aim III was to collect and compare gait parameters to investigate changes in weight distribution and gait kinetics. Gait parameters were collected pre- and at various time points post-surgery using a force plate and motion capture system to observe changes and trends. There was no significant difference in the load distribution between the front legs or the load distribution between the back legs pre-surgery. Loading of the amputated leg decreased by over 50% in all animals, while the intact-side front leg compensated by increasing weight bearing. Muscle contracture was present in the semitendinosus and semimembranosus muscles of the amputated leg of all animals and was measured by an overall decrease in the maximum angle between the prosthetic and the ground.

Results from this pilot study will be used in developing and implementing a full animal study for this novel transcutaneous osseointegrated (TOI) solution to the socket prosthetic. This animal model should become the standard for the future testing of TOI prostheses as an option for lower-limb amputees.

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NOTATIONS AND CONVENTIONS (ALPHABETICAL)

α – Internal Consistency

A/P – Anterior-Posterior

AB – Acinetobacter Baumannii

ABL – Amputated Back Leg

AFL – Amputated side, Front Leg

AK – Above-the-Knee

amp – amputated

BK – Below-the-Knee

cm – centimeter

DT – Differential Torque MZ

ED – Endosteal Diameter

EO – Equipment Operator

FDA – Food and Drug Administration

FX – Force in X

FY – Force in Y

FZ – Force in Z

GC – Gait Cycle

GRF – Vertical Ground Reaction Force

H_2O – Water

H_2O_2 – Hydrogen Peroxide

HO – Heterotopic Ossification

hr – hour

HRQL – Health Related Quality of Life

IACUC – Institutional Animal Care and Use Committee

IBL – Intact Back Leg

IFL – Intact side, Front Leg

IM – Intramuscularly

iso – isoflurane gas

IV – Intravenously

KP – Klebsiella Pneumonia

LAR – Laboratory Animal Resources

LL – Lower Limb

LMM – Linear Mixed Model

M/L – Medial-Lateral

m/s – meters per second

Max – Maximum

mg – milligram

Min – Minimum

ml – milliliter

mm – millimeter

MX – Moment about X

MY – Moment about Y

MZ – Moment about Z

OD – Outer Diameter

P – Periosteal Diameter

PRE – All time pre-op; Weeks -4 through -1 pre-op

Pre-op – Pre-Operation

Post-op – Post-Operation

Q1 – Quarter 1; Weeks 1-4 post-op

Q2 – Quarter 2; Weeks 5-8 post-op

Q3 – Quarter 3; Weeks 9-11 post-op

R – Pearson Correlation Coefficient

S1 – Velocity 1: 0.500-0.744 m/s

S2 – Velocity 2: 0.750-0.999 m/s

S3 – Velocity 3: 1.000-1.244 m/s

S4 – Velocity 4: 1.250-1.499 m/s

S5 – Velocity 5: 1.500-1.744 m/s

S6 – Velocity 6: 1.750-1.999 m/s

S7 – Velocity 7: 2.000+ m/s

SI – Surgical Incision

SM – Stenotrophomonas Maltophilia

TOI – Transcutaneous Osseointegrated

US – United States

%GC – Percent of Gait Cycle

%WD – Percent Weight Distribution

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Chapter 1. Introduction and Background

1. INTRODUCTION

The number of people living with an amputation in the United States (US) surpassed 1.7 million in 2008 and is projected to breach 2.2 million by 2020.[6-9] As reported in the last census, lower-limb (LL) amputations made up 61.46% of all amputations in 1996[6] and is projected to increase to 58,000 per year by 2030.[10] With such a high number of patients living in the United States with a lower extremity amputation, there are surprisingly very few options in terms of modes of attachment of the prosthesis to the body.

1.1 SOCKET PROSTHETIC AND ITS PROBLEMS

The socket prosthetic is used by LL amputees to replace the amputated limb and designed to mimic the function of an anatomical leg.[11, 12] While there are minor variations in style and purpose, all socket prosthetics have the same basic anatomy [Figure 1] with the socket being the most widely used mode of attaching the prosthetic to the body. Each socket is custom fit to the patient and slips over the distal end of their stump, starting with application of the liner [Figure 2]. The socket can be made of a variety of plastics or carbon fiber, none of which allow the skin to breathe. When the skin is not allowed to breathe, the temperature inside the socket increases and causes the skin to sweat. In fact, 72% of patients have reported problems with sweating.[13, 14] Consistent with socket comfort being reported by patients as a major concern, a surprising number of skin-health issues arise from this environment created within the socket. [19-21] Table 1 lists the components of a standard socket prosthetic and problems associated with each.

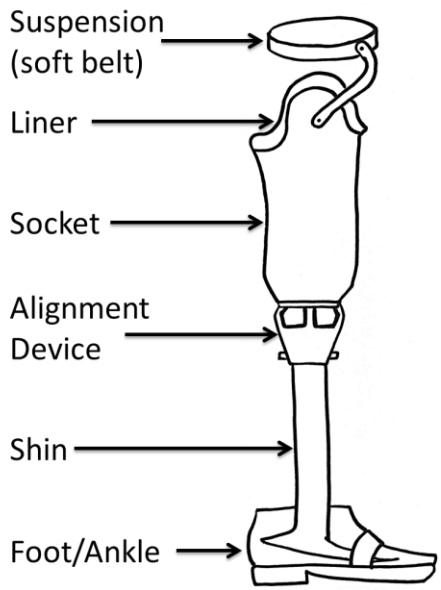

Figure 1. Socket prosthetic diagram with labels.

Figure 2. Liner application to stump.

Image adapted from McKinney Prosthetics (2014)[2]

Table 1. Socket Prosthetic Anatomy Descriptions, Problems, and Resulting Consequences

Prosthetic Part	Function	Problem	Results in…
Suspension (soft belt)	Anchors prosthetic to body	Needs multiple readjustments throughout the day	Unreliability of the prosthetic being securely attached; causes mobility difficulties for the patient[27, 29, 31, 44, 45]
Liner	Absorbs sweat; acts as a buffer between socket and skin to limit rubbing	Does not decrease amount of sweat produced; creates constantly moist environment, resulting in significant comfort problems for the patient[17, 33]	Irritation and swelling, pressure sores, rashes and blisters, pain and discomfort[17, 18, 26, 27, 29, 31, 37, 54, 59]
Socket	Encompasses residual limb and distributes load	Rigid – does not move with patient; made of a variety of plastics or carbon fiber, none of which allow the skin to breathe.	Rubbing of socket against liner and skin; increased temperature inside the socket, causes skin to perspire
Alignment Device	Attaches and aligns socket and prosthetic	Detachment process is lengthy	
Shin and Foot/Ankle	Support; Transfers load from ground to socket	------	

During normal human gait, the skeleton is directly loaded through a thin layer of soft tissue on the bottom of the foot. This load transfer provides direct sensory feedback to the brain so that one is always aware of the location of their foot in relation to their body. This awareness allows them to constantly adjust the weight distribution in order to maintain balance over varying terrains.[22, 23] However, in patients with LL amputations, this direct feedback is not so easily attainable. Unlike the load transfer through the skin at the bottom of a foot to the skeleton of an intact limb, the load of a lower-limb amputee is transferred through the prosthetic to all surfaces in contact with the stump. The outermost surface is the skin, but the main tissue that absorbs the impact is the subdermal soft tissue, namely the quadriceps and hamstrings in above-the-knee (AK) amputees and gastrocnemius, or calf muscle, in below-the-knee (BK) amputees. This results in the direct loading of soft tissue and has been reported by amputees as very painful.[12, 18, 24, 25]

Another problem that occurs during ambulation is the distal end of the amputated bone pushing directly into the muscle and soft tissue with every step. This has been known to cause small pieces of bone to break off and become lodged in the soft tissue which can result in irritation and tenderness of the local muscles and soft tissue.[13] It can also cause heterotopic ossification (HO), or bone formation outside of the skeleton, which is present in over 50% of amputees.[26-28] This HO is reportedly very painful to patients and difficult to remove. Patients have also reported difficulty donning the prosthetic as well as discomfort while sitting with the prosthetic attached.[13, 29]

1.2 PROBLEM SIGNIFICANCE

Due to the pain and lack of convenience of this socket prosthetic, patients can become inactive, sitting for the majority of their day, and ultimately don't wear the prosthetic often or stop using it altogether. This decrease in mobility leads to a more dormant lifestyle, and has been shown to negatively affect the patients' health-related quality of life (HRQL).[14] Of all lower extremity amputations, 75% of them occur in patients aged 65 years and older, and this population is projected to double over the next 50 years. [30, 31, 32] With that in mind, developing a less problematic alternative to the socket is even more critical.

1.3 AN ALTERNATIVE SOLUTION TO THE SOCKET PROSTHETIC

An answer to the problems caused by the socket has been pioneered and implemented in over 250 amputees in Europe since 1990.[5, 11, 13, 29, 33-37] This new technique involves a transcutaneous (through the skin) osseointegrated (anchored in bone) (TOI) implant that allows for attachment of an external prosthesis and therefore direct skeletal loading.

1.3.1 Transcutaneous Osseointegration

The term 'osseointegration' was first introduced in the 1950's by Rickard Brånemark in Gothenburg, Sweden, and is defined in theory and practice as "continuing structural and functional coexistence, possibly in a symbiotic manner, between differentiated, adequately remodeled, biologic tissues and strictly defined and controlled synthetic components, providing lasting, specific clinical functions without initiating rejection mechanisms."[37] In practice, there is osseointegration where there is "an anchorage mechanism whereby non-vital components can be reliably and predictably incorporated into living bone and that this anchorage can persist

under all normal conditions of loading."[37] He showed how titanium chambers could be permanently fused with living bone and coined the term 'osseointegration' to describe this fixation.[37] Upon realization of this fixation, Brånemark integrated titanium screws into bone for long-term support of dental prostheses that were transcutaneous at the gum.[37, 38] Over the years, the application of TOI implants has been used in oral surgery, hearing aids, finger prostheses, and thumb amputations.[37] More recently, the application of TOI implants has extended to the field of orthopedics, specifically LL amputations.[37]

1.3.2 TOI Implant for Application in LL Amputations

In application of TOI implants to LL amputations, a procedure has been developed and implemented in over 250 patients to date.[11, 13, 37, 39, 40] Dr. Horst H. Aschoff, a surgeon in Lubeck, Germany, is one of the surgeons who has performed this procedure, and it is Dr. Aschoff's procedure off of which this study was modeled. He has developed an implant system called Endo-Exo that is porous only on the surface that is placed inside the bone and is otherwise polished smooth [Figure 3, Figure 4].[5] This smooth surface prevents the skin from attaching and instead promotes down growth of the skin to form a stoma. Instead of adhering to the implant to form a seal at the skin-to-implant interface, the skin is encouraged to grow proximally, adhering to the bone.[5, 33] In theory, this would seal the skin to the bone, permanently providing a barrier between the outside world and the internal tissue. However, a definitive seal has not yet been achieved and consequently leaves the patient with a wound that is not entirely closed for the remainder of their life, causing the patient to be susceptible to

infection. Preventing infection, increasing wound healing, and achieving a permanent seal must be investigated.

Figure 3. Dr. Aschoff's Endo-Exo System.

Image adapted from Aschoff (2010)[5]

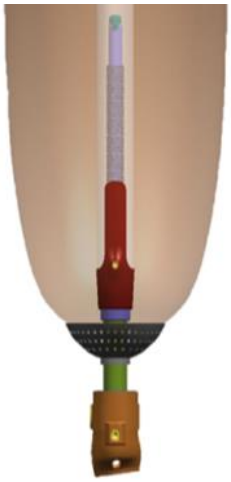

Figure 4. Endo-Exo System, implanted example.

Image adapted from Eska America (2008)[3]

1.4 TOI SURGICAL PROCEDURE FOR LL AMPUTEES

This pilot study was designed to develop an animal model in order to investigate the shortcomings of the TOI prosthesis, specifically wound healing and infection at the skin-to-implant interface. With a long-term goal of obtaining FDA approval, an animal model is needed for the current human procedure of TOI applications in AK amputees. The surgical procedure

used in this animal study was designed to reproduce the human procedure performed by the aforementioned Dr. Aschoff. This operation utilizes the Endo-Exo implant system and is divided into two surgical procedures. During the first surgery, the tibial canal is reamed in a retrograde fashion, and the intramedullary component of the device is implanted. The wound is then closed and sutured over the end of the internal prosthesis [Figure 5]. The bone is left to heal and grow into the implant.

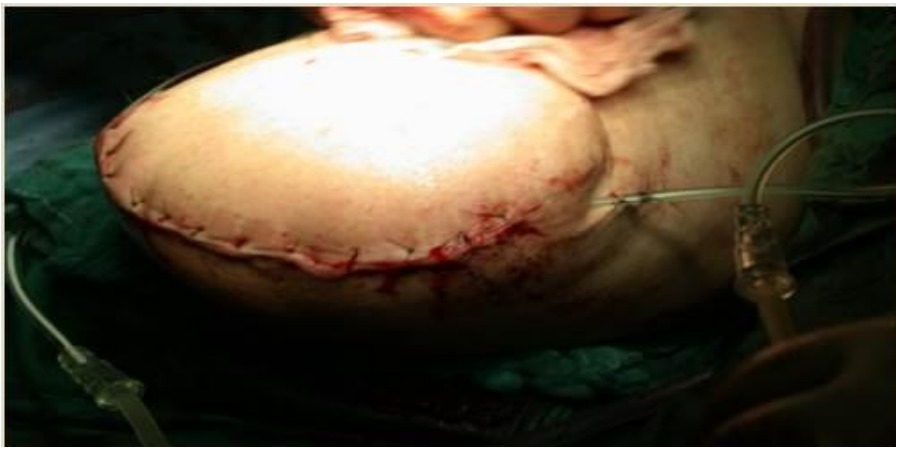

Figure 5. Human stump immediately after first surgery. Incision site, no stoma.

Image adapted from Eska-America (2008)[3]

After a 6-8 week healing period, the second operation is performed. In this out-patient procedure, Aschoff creates a stoma by cutting a circular incision through the skin and soft tissue over the end of the intramedullary component and attaches a transdermal coupler, which is later used for connection of a prosthetic [Figure 6, Figure 7]. The incision has a diameter slightly larger than that of the coupler. Using a coupler with a smooth surface has shown to have a lower rate of superficial skin problems than when using a coupler with a textured surface.[5, 34, 35, 37, 41]

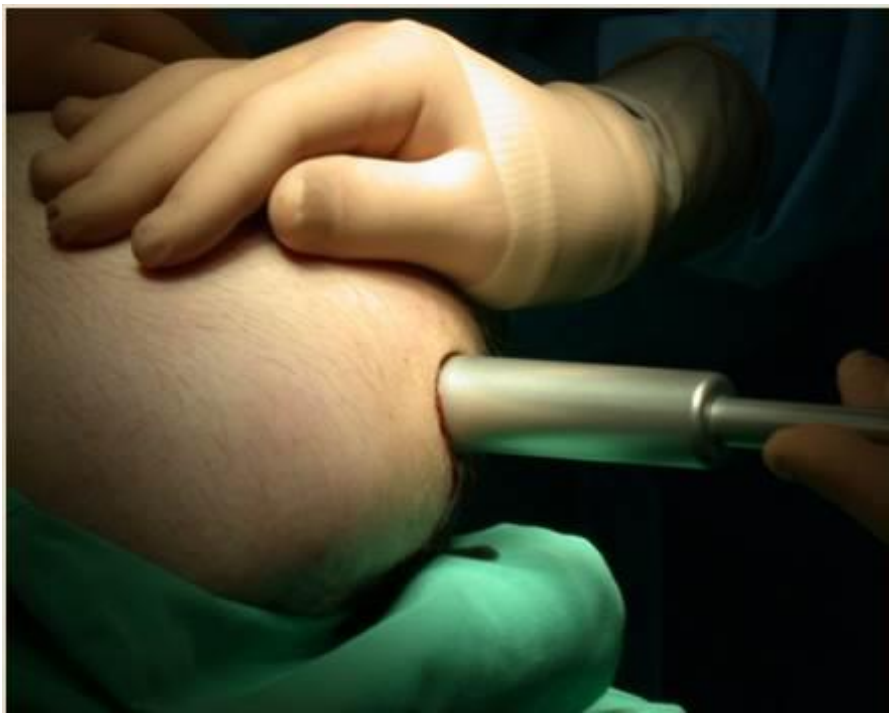

Figure 6. Human procedure, creating the stoma using a surgical punch.

Image adapted from Eska-America (2008)[3]

Figure 7. Human stoma showing distal end of implant, immediately post-surgery.

Image adapted from Eska-America (2008)[3]

1.4.1 Post-Surgical Care

After Aschoff performs the second surgery, the patient must maintain and clean the wound. The current protocol for post-surgical wound care instructs the patient to clean the wound with soap and water twice daily and replace the dressings with new ones at each cleaning. Because this stoma is an open wound for the remainder of the patient's life, there is always discharge, so the dressings must be changed frequently to prevent infection [Figure 8].

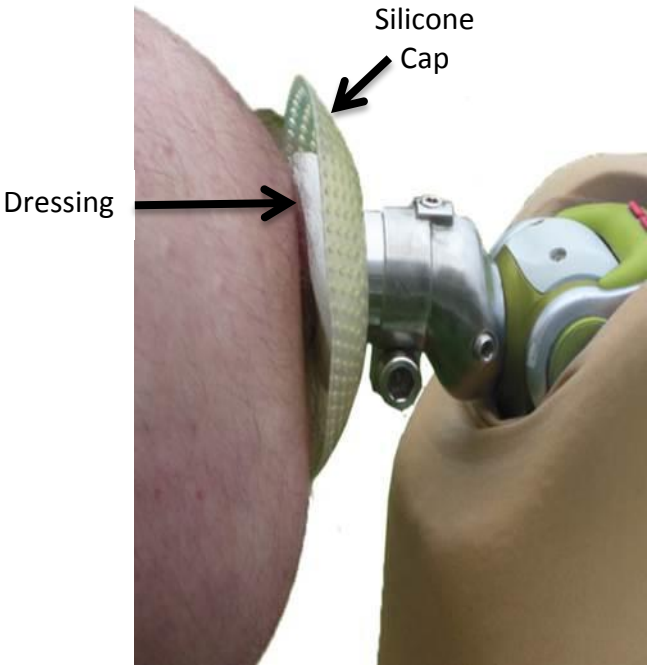

Figure 8. Human stoma with dressing and silicone cap; prosthetic attached.

Image reproduced from Eska-America (2008)[3]

1.4.2 Pros and Cons

While this TOI prosthesis has its pros and cons, the presence of an open wound has contributed to high rates of infection. In combination with a lack of antibiotics used in Europe, patient compliance has also played a large role in these high infection rates. Implant infections

are present in 5% of patients within six months post-surgery and in 18% of patients within two to three years.[42] However, the advantages to this TOI procedure heavily outweigh the disadvantages. Transcutaneous osseointegration as an alternative to socket prostheses has been proven to increase the HRQL of recipients.[11, 43, 44]

In two separate studies performed in 2008[11] and 2011[18], a total of 31 TOI patients were asked about how they experienced living with their osseointegrated prosthesis as compared to their previous socket prosthesis.[18] Eighteen patients answered two self-report questionnaires both before the procedure and at a 2-year follow-up[11]; the remaining thirteen patients were interviewed in-depth once at varying time points 3 to 15 years after their procedure.[18] The patients were amazed at how both their bodies and their brains have adopted the prosthetic as an extension of their body: "*I don't think about having the prosthesis in that it doesn't feel like a prosthesis… It has come so far that the brain has also gradually begun to believe that I have a real leg.*"[18] *"It's much more integrated than it was with this old prosthesis* (socket prosthesis) – *it becomes a part of you."*[18] The socket prosthetic doesn't come nearly as close as the TOI prosthetic to feeling like a real leg: *"I can feel that it's* (TOI prosthetic) *not as good as a healthy leg, but ti's far more normal than the old one* (socket prosthetic)*… This is perhaps 70% as compared to a real leg and a real leg being 100% and an old prosthesis is perhaps 25%."*[18]

One added benefit of direct fixation of the prosthetic to the bone via a TOI implant is the improved sensory feedback, which can improve locomotion.[43, 45] *"…it's anchored with material from my own body… It's very concrete. As opposed to a traditional prosthesis that is slipped on to the outside of the body. But here I can feel when I put the foot down, so that I can*

feel the shock throughout the body, not in an unpleasant way but I feel it and it gives me a positive experience of my body as a whole."[18] The more noticeable advantages are the physical and prosthetic benefits, including but not limited to easy attachment/detachment allowing the user to quickly and efficiently swap out one prosthetic for another; eradicating the socket so that the skin can breathe; eliminating soft tissue loading which results in fewer sores, less inflammation, and decreased HO; more comfortable; decreased pain results in increased activity, which in turn results in a healthier patient and increased HRQL.[11, 14, 43]

Overall, the HRQL was significantly increased with the TOI prosthesis.[11] *"The other prosthesis ruled my life, it was my master in a way, it's inevitable… it affected my mood and my interest in doing things that I knew would demand an extra effort. You had to weigh the pros and cons and that's all gone now. Now it's actually me… I am in command and not the* [socket prosthetic] *and that's a big difference."*[18] Ultimately, they feel like they have their old leg back, and that alone increases their HRQL dramatically.[11, 13, 18]

1.5 ADOPTING TOI IMPLANTS FOR APPLICATIONS IN LL AMPUTEES IN THE US

In order to make this procedure accessible to patients within the US, there are many steps that must be completed before FDA approval can be granted. These steps begin with animal research. Due to a high infection rate of 8-19% in human patients in Europe, it must be proven that this procedure can be successful in animals before it can be performed in humans.[20, 33, 42, 46]

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Chapter 2. Study Design

2.1 MODEL SELECTION

Researchers have tried to improve the wound healing at a skin-to-transcutaneous-implant interface in other large animals, but none have been successful in developing an animal model for human wound healing in this application. Two studies that attempted this very thing are Bloebaum's sheep study and Fernie's pig study.[47, 48]

In 2012, Bloebaum developed an animal model to "evaluate the efficacy of a porous-coated titanium subdermal barrier to achieve skin-implant integration."[49] An amputation of a front limb and implantation of a percutaneous device was performed on 23 sheep: nine served as the control group and received smooth titanium subdermal barrier constructs, while the remaining 14 were fitted with porous-coated titanium subdermal barrier constructs. At the nine-month endpoint, he concluded that porous-coated titanium *may* have the ability to maintain a "healthy, biologically attached epithelial barrier at the skin-implant interface in load-bearing animals."[49] However, while something *may potentially* have an *ability*, they did not prove that it was successful. Not only did Bloebaum fail to provide a successful epithelial seal, there is no evidence that sheep are a good model for human wound healing because they are covered almost entirely with a wool coat. These hair follicles play an important role in wound healing of sheep and other large animals, and therefore their healing process is not comparable to wound healing in humans.[1, 50]

Fernie et al. conducted a series of experiments on 14 pigs to study the tissue attachment to a velour covering and to test bone adhesion to a porous metal surface coating of a percutaneous implant.[47] They amputated a hind limb and inserted an implant with a porous

metal coating on the intramedullary part and a nylon velour or Dacron velour fabric covering at the soft tissue-implant interface. While fixation of the bone was achieved, the velour was unable to maintain an epithelial tissue seal at the soft tissue interface.[47] They concluded that, while skin does grow into nylon or Dacron velour, the epithelial seal cannot withstand the stresses endured at the amputation site.[47] A constant and secure epithelial seal was not developed, which led researchers, specifically Bloebaum, to search for another medium for skin ingrowth.

Pigs have been used as a model for human wound healing due to the unique anatomical and physiological similarities between pig and human skin.[1, 51] Humans and pigs have a similarly thick epidermis, with the human skin having a thickness of 50 to 120 µm and the pig skin 40 to 140 µm.[1] Pigs and humans share the characteristic of having sparse body hair. Unlike many animals, the body hair of both the pig and the human "progresses through the hair cycle independently of neighboring follicles. This is important as adnexal structures, including hair follicles, play an important role in re-epithelialization."[1, 47, 52] This process is key to wound healing because, instead of relying on wound contraction for wound closure like most small animals, man and swine rely on re-epithelialization to close partial-thickness wounds.[1] Figure 9 "visually illustrates the histological similarities between pig and human skin."[1] Therefore, pigs were chosen as the animal for this study.

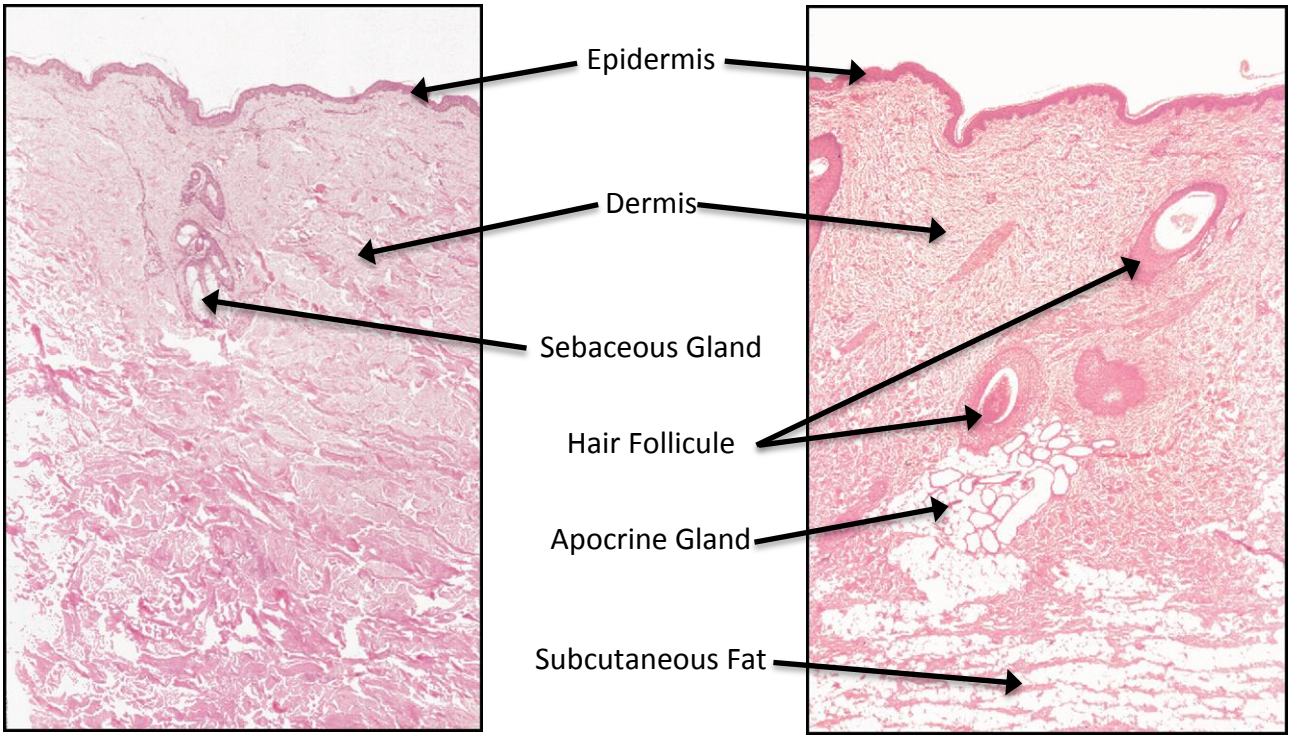

Figure 9. Comparison of histological features from swine and human skin. H&E stained section taken through comparable portions of the dermis in both tissues.
Images reproduced from Sullivan et al. (2001) [1]

Four intact male Yucatan mini pigs age 8.4 ± .4 months and weighing 38 ± 2kg from Sinclair Bio Resources, LLC in Columbia, Missouri were used for this study. The Yucatan breed was chosen for their lack of hair, their fast growth rate to maturity, and their weight. Their skin has little to no hair, which was additionally beneficial for studying the wound site. The lack of hair allowed for easy cleaning, monitoring, and observation of the stoma throughout the study. Stability of the model required skeletally mature pigs. Compared with domestic swine whose weight can reach 306kg when fully grown, Yucatan mini pigs weigh between 57kg and 80kg when skeletally mature, which not only allows for easier handling, but also more closely represents the weight of adult humans.[52] There is no significant difference in the growth rate

of females versus males, so males were chosen because they were less likely to micturate on the wound dressings and soil the stoma site. More information on Yucatan Mini Pigs can be found in A1. Yucatan Miniature Swine Information.

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas Medical Center in Kansas City, Kansas. This pilot study was completed on Pigs 1 and 2 (Fall 2012 - Figure 10) before continuing on Pigs 3 and 4 (Fall 2013 - **Error! Reference source not found.**). The duration of the study for each animal was 8 weeks, 5 weeks, 11 weeks, and 10 weeks respectively.

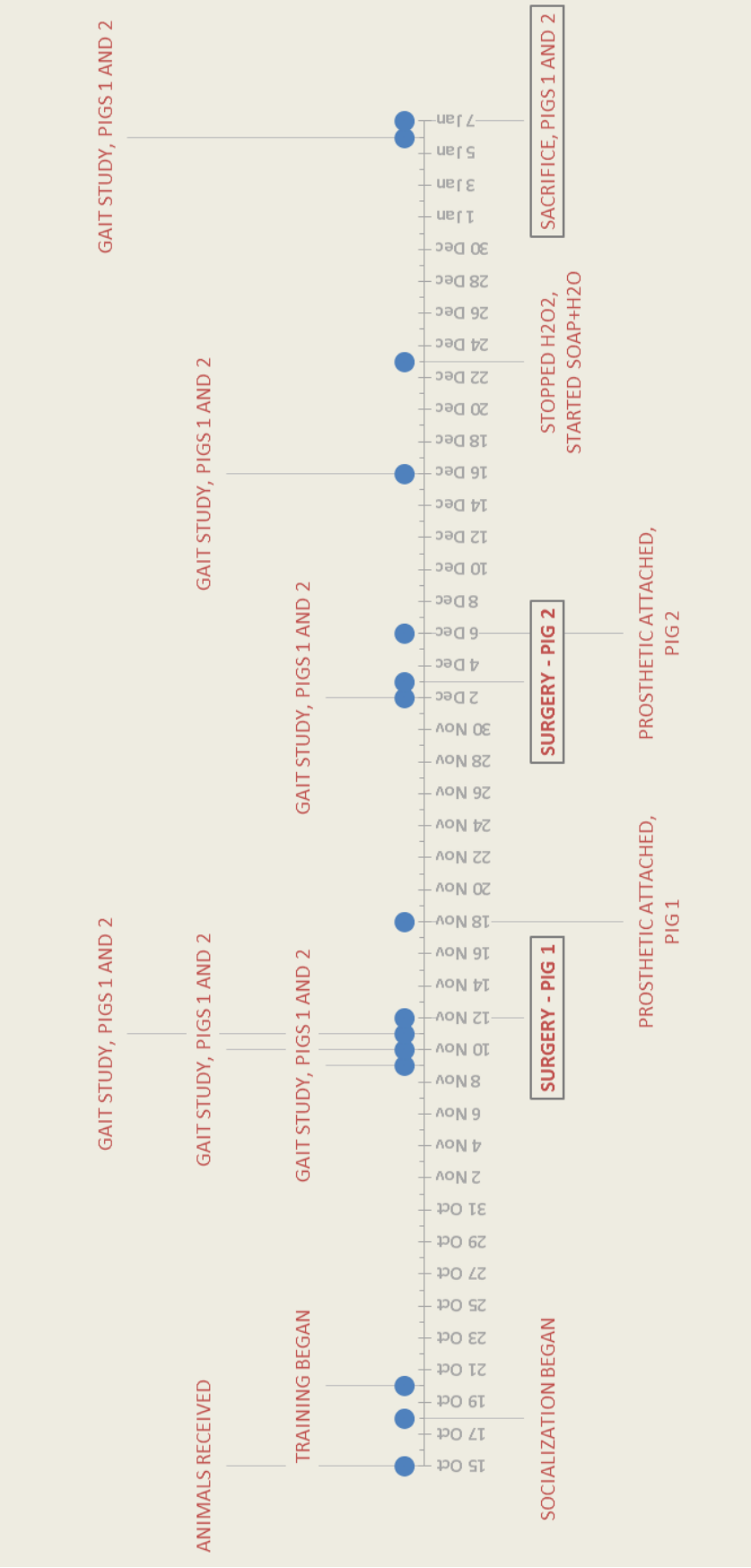

Figure 10. Timeline - Pig 1 (8 weeks) and Pig 2 (5 weeks); 2012.

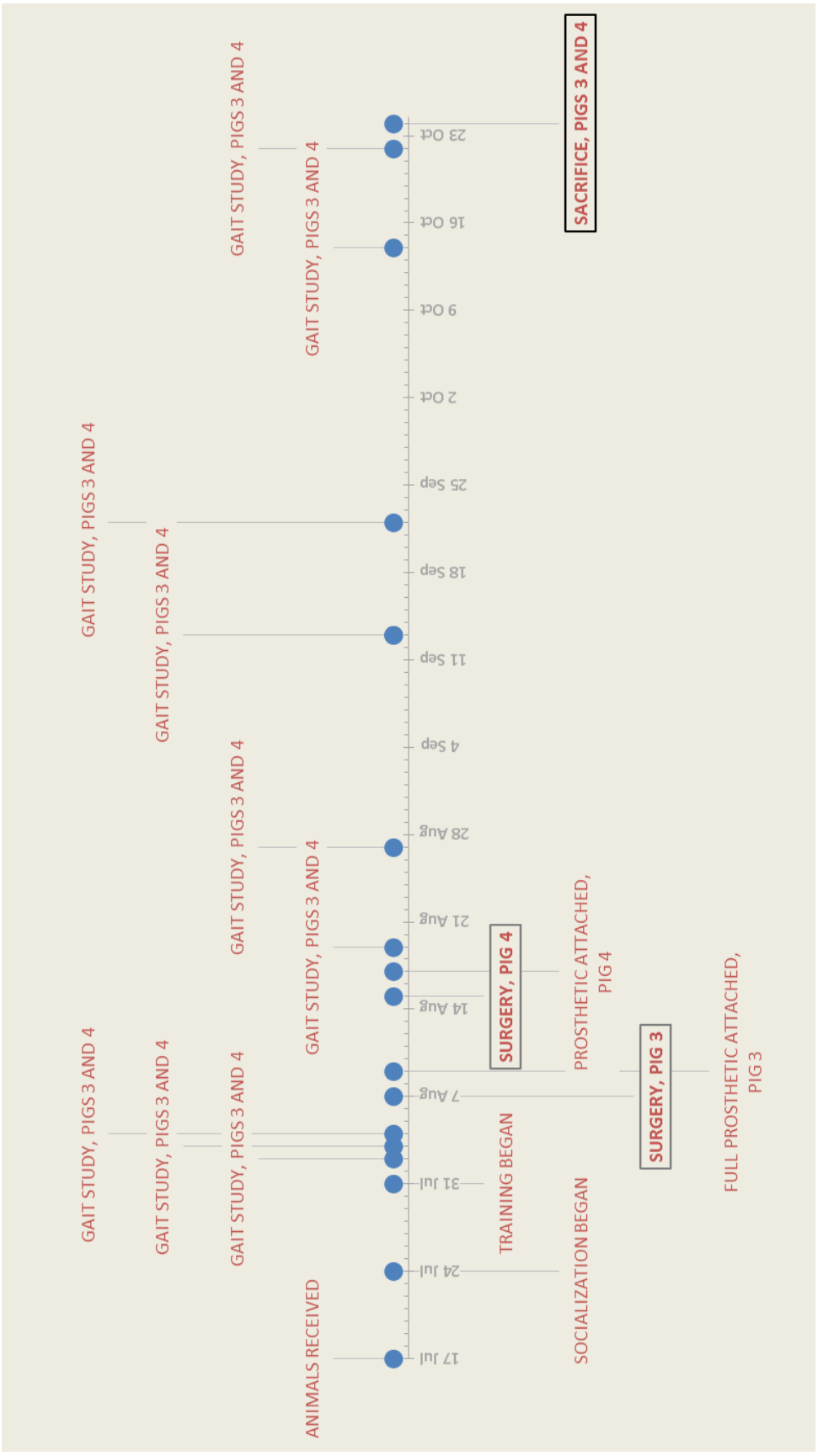

Figure 11. Timeline - Pig 3 (11 weeks) and Pig 4 (10 weeks); 2013.

2.2 TRAINING AND HOUSING

In accordance with the IACUC regulations, the animals had an acclimation period of seven days once they arrived at our on-site Laboratory Animal Resources (LAR) facility. During and after that acclimation period, we allowed the animals to get better acquainted with our presence: we spent time in their pens, provided socialization, and allowed them to get more familiar with wearing a harness and leash. Food items, including but not limited to Oreo cookies, were used as reward during training and exercises for each animal. Training involved walking across the force plate platform and walking around wearing the harness with the leash attached. The pigs were housed together in a connecting pen pre-surgery and housed in separated pens post-surgery to prevent any damage to the wound. Each pig received daily enrichment that varied from listening to the radio, playing with toys in their pens, and human interaction.

2.3 SPECIFIC AIMS FOR THIS PROJECT

1. The first specific aim of the project was to develop, execute, and validate a hind leg amputation on four pigs, modeled after an above-the-knee amputation on humans. I assisted in the training and planning of the surgical process as well as adapting the human procedure to the animal procedure. I worked with the orthopedic surgeon on the practice cadavers, developed the appropriate surgical procedure with my team, and assisted in surgery.

2. The second specific aim was to prevent infection while monitoring and promoting wound healing. I performed post-surgical procedures of cleaning the wound, changing the dressings, and monitoring the bacterial growth.
3. The third specific aim was to collect and analyze both visual and numerical data on gait and weight distribution repeatedly both pre-operation (pre-op) and post-operation (post-op) to determine how well the animals adapted to the amputation, as well as the potential impact the amputation had on the other joints. I constructed the materials needed for this study in addition to writing all of the code to capture and analyze all data. I performed all of the gait studies on every animal throughout the entire study. Gait data were collected and analyzed and allow us to see how the amputation affects gait kinematics and kinetics throughout the study.

2.4 STUDY ENDPOINT

The pigs were euthanized as per protocol at 5, 8, 10, and 11 weeks post-op.

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Chapter 3. Specific Aim I

Develop, execute, and validate a hind leg amputation on four pigs, modeled after an above-the-knee (ATK) amputation on humans.

3.1 METHODS AND MATERIALS

3.1.1 ADAPTATION TO THE ANIMAL MODEL

Unlike the human procedure, which is split into two separate surgeries to allow the bone to grow into the implant and securely fix the implant in place, the animal procedure was modified to combine both human procedures into one surgery. In the human procedure, the first surgery involves insertion of the implant and complete wound closure.[5] Six to eight weeks later, a second surgery is performed during which the surgeon inserts the transcutaneous piece of the device.[5] In the animal procedure, both of these steps were combined into one surgery in accordance with IACUC guidelines that animals only undergo one major surgery.

Additionally, the anatomy of a swine hind leg in reference to the torso is of a slightly different arrangement than that of a human leg [Figure 12, Figure 13]. The femur in humans is surrounded by soft tissue, namely the hamstrings and quadriceps. In order to accurately model an AK amputation, the amputation in the pigs had to be through a bone that would be adequately surrounded by soft tissue and still be accessible for the research. The femur of the pig is located almost entirely within the torso area, whereas the tibia is easily accessible and has a sufficient amount of soft tissue to adequately model an AK amputation in humans. Therefore, a transtibial amputation was considered analogous to the transfemoral amputation in humans for the purposes of this study.

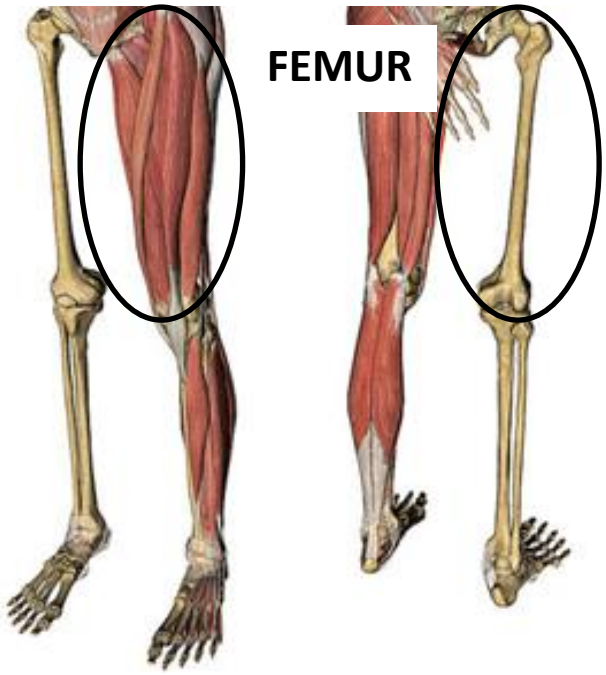

Figure 12. Human skeleton and muscle representation.

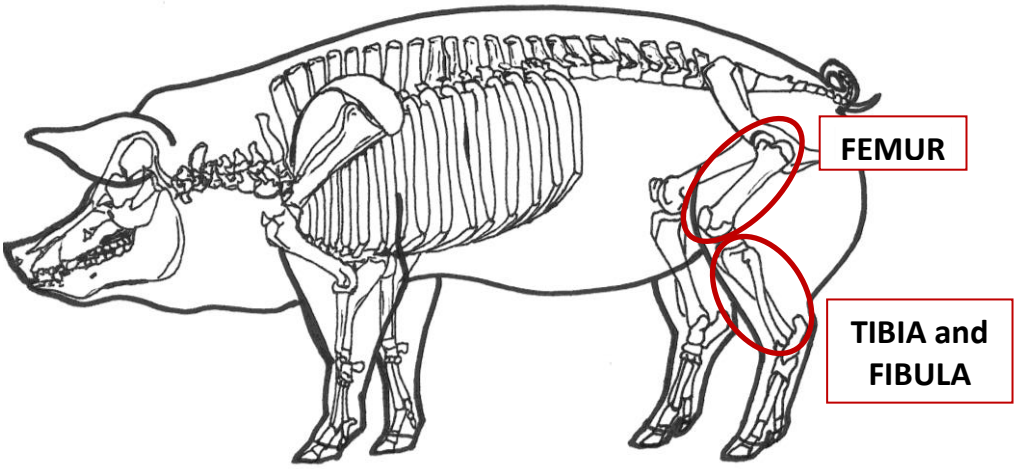

Figure 13. Swine skeleton imposed on a picture of a swine.

3.1.2 IMPLANT DESIGN

Implant design and manufacture were primarily executed by another student in the KUMC Orthopedic Research Laboratory.{Colbert, 2013 #145} When designing an intramedullary implant, locally high stresses must be avoided to prevent bone necrosis.[53] Conversely, "the implant must also be designed to allow stress patterns within the bone that will prevent its resorption."[53, 54] Additionally, we needed to ensure bone fixation with the design of the implant[5]. Therefore, with the exception of the most proximal tip, the entire intramedullary portion of the implant was threaded, and cutting flutes were incorporated into approximately 60% of the threads [Figure 14]. These cutting flukes were used to provide faster and easier insertion of the implant into the medullary canal during surgery. The dimensions for each implant were determined by x-rays [Figure 15].

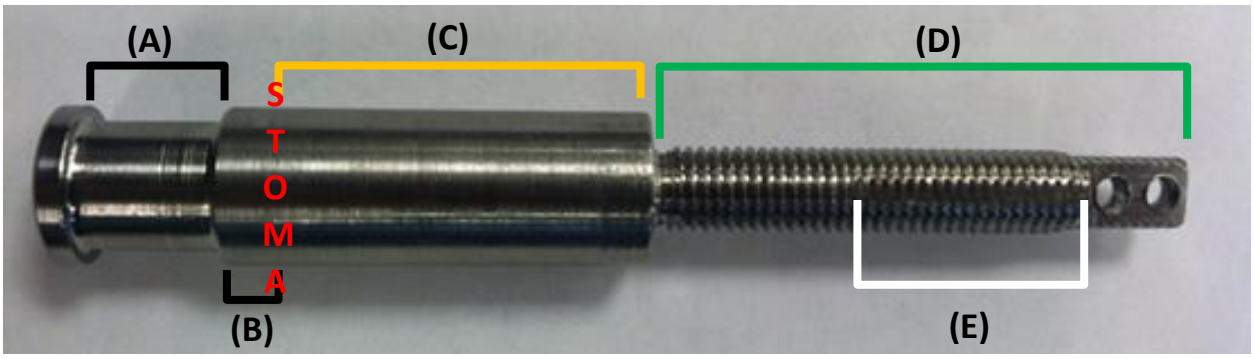

Figure 14. Implant: location anatomy. (A) prosthetic block; (B) cup and dressings; (C) skin and soft tissue; (D) bone; (E) cutting flutes.

Figure 15. Anterior-posterior x-ray with example medial-lateral bone measurements of swine tibia. Green = periosteal diameter; Purple = endosteal diameter.

3.1.2.1 Implant Sizing

Using x-rays taken in the medial-lateral (M/L) direction, anterior-posterior (A/P) measurements of the bone were found. Likewise, x-rays taken in the A/P direction were used to calculate the M/L measurements. The most proximal and distal ends of the medullary cavity were determined visually, and the radius of both the endosteum and periosteum were

calculated at 1cm intervals on both x-rays. Those dimensions were then plotted in Excel and used in Equation (1) to define the necessary range of dimensions of the implant to ensure a fixed anchoring in the bone once implanted. The implant was made of Grade 5 Ti-6Al-4V and was manufactured in-house.

$$OD = [(P - E)\ x\ 0.53] + E$$

OD = Outer Diameter, P = Periosteal Diameter, E = Endosteal Diameter

3.1.2.2 Implant Anatomy

Figure 14 shows the implant with labels describing where each part of the implant was located once implanted. There was a notch at the distal end (A) where the prosthetic block, to be described in detail later, was attached. The silicone cap alternative ("cup") and wound dressing were adjacent to the stoma (B), and the skin and soft tissue (C) surrounded the solid piece of Ti(tanium) up to where the implant was flush with the bone (D). The threads (D, E) were located in the medullary canal of the tibia. The two holes at the proximal end of the implant were implemented in the second, third, and fourth implants to provide better bone cement integration and fixation with the implant.

3.1.3 SURGERY

A4. Surgery lists all drugs used throughout the study. The surgical procedure was performed by an orthopedic surgeon and was the same for each animal.

Approximately ninety minutes pre-operatively, 100 mg/ml Ketamine (general anesthesia) and 5 mg/ml midazolam (sedative) were injected. A 100 mg/hr Fentanyl patch (opioid analgesic - pain management) was placed on the dorsal neck. Additionally, 9.3 mg/ml Buprenorphine (opioid analgesic) and 1.5 ml Atropine (sedative) were injected intramuscularly (IM). A spinal block was performed by injecting 4.9 ml Buprivacaine (narcotic analgesic) into the spinal cord. A 400 mg Cephazoline (antibiotic) injection was given intravenously (IV). Two swabs were collected during pre-operative preparation: one on the lateral skin of the leg to be amputated, one on the medial skin of the leg to be amputated. All swabs were used for Specific Aim II.

Once the pre-operative preparation concluded, the animal was brought to the surgical suite and placed in the supine position. Pre-operative x-rays were taken [Figure 16]. After sterilization of the leg to be amputated, two additional swabs were collected from the same locations as during pre-operative preparation. A longitudinal incision was made anteriorly approximately 3cm proximal to the ankle joint [Figure 17]. The incision extended proximally in a fish-mouth manner to allow for appropriate coverage at the conclusion of the case. The incision was extended distally around the heel-pad and calcaneus. The heel pad and surrounding intact soft tissue would be later used as the skin flap over the distal end of the stump. The soft tissue was elevated off of the bone to be amputated along the incision. Once

neurovascular structures were tied off and tendons were detached, the tibia was cut 3cm proximal to the anterior skin incision. The fibula was cut proximal to the tibia. The distal amputated leg was then discarded.

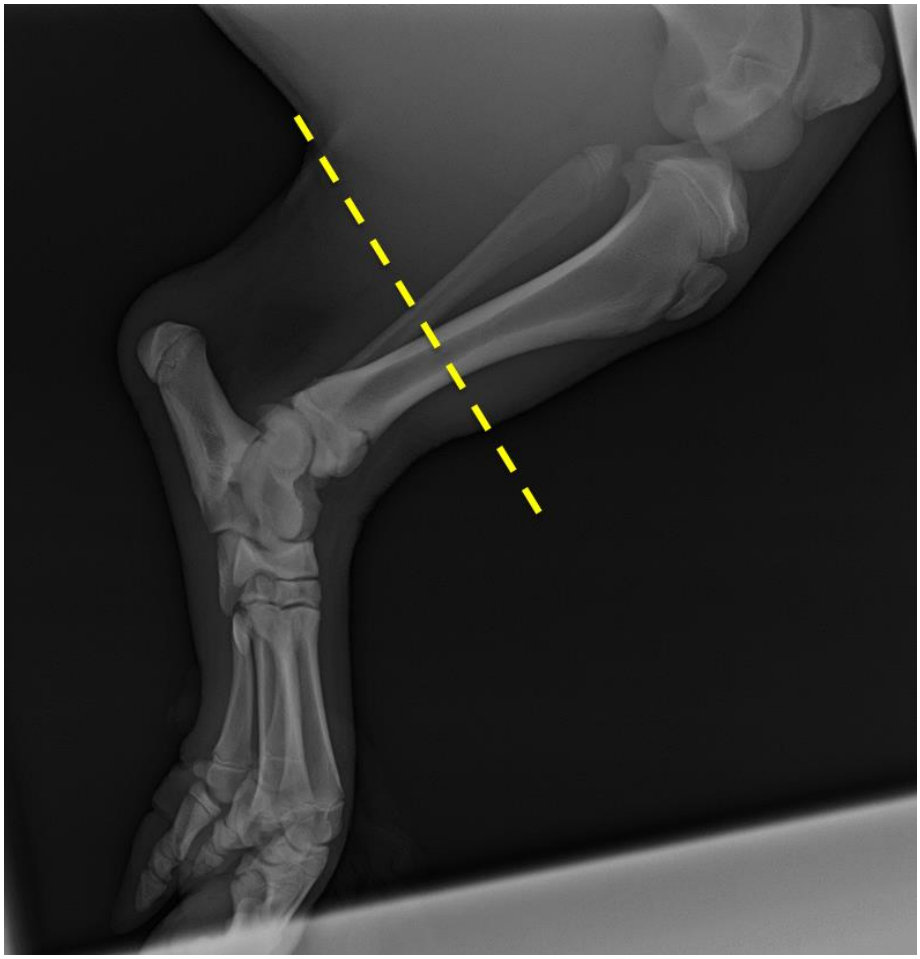

Figure 16. Pre-op x-ray: the yellow dash line shows approximate location of amputation for each animal.

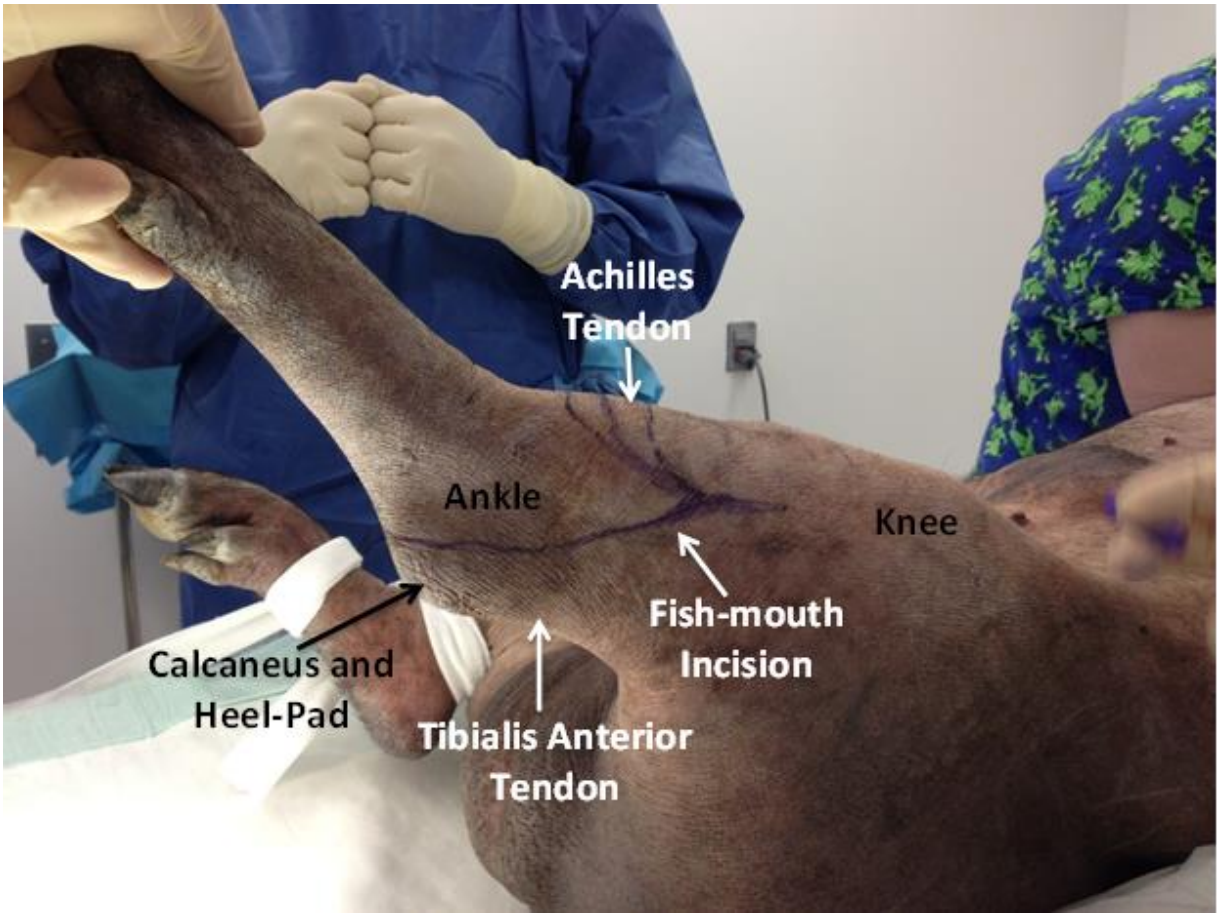

Figure 17. Surgical incision and anatomy.

Next, the medullary canal was reamed first with a 7mm drill bit then with an 8mm drill bit [Figure 18]. The sizes were chosen based on the initial diameter of both the medullary canal and the compact bone as determined by initial x-rays [Figure 15]. The implant was then inserted partially into the tibial canal to allow the cutting flutes to initiate [Figure 19]. This created threads along the inside of the tibia [Figure 20], which facilitated a more precise, expedited, complete insertion of the implant once the wound was closed.

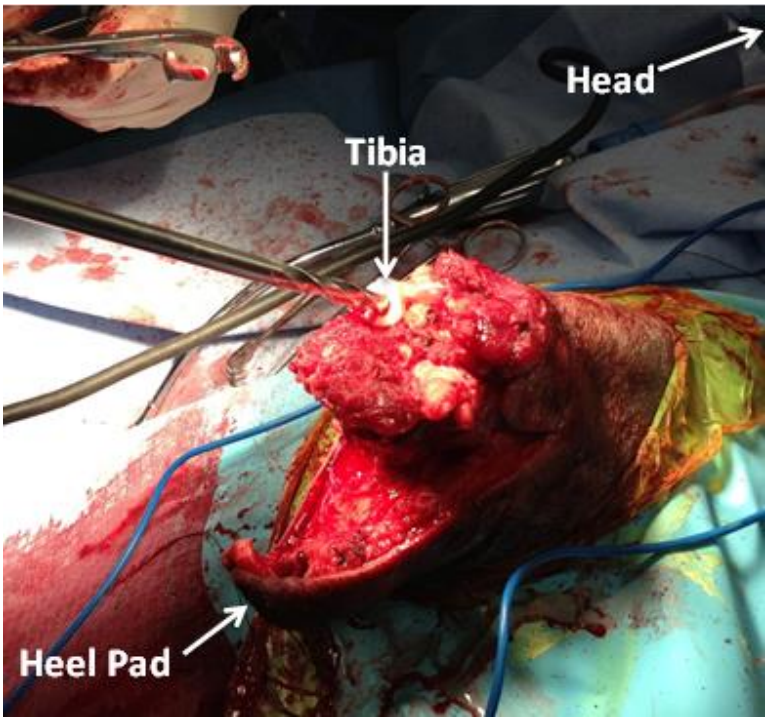

Figure 18. Mid-surgery, reaming tibia with 8mm drill bit.

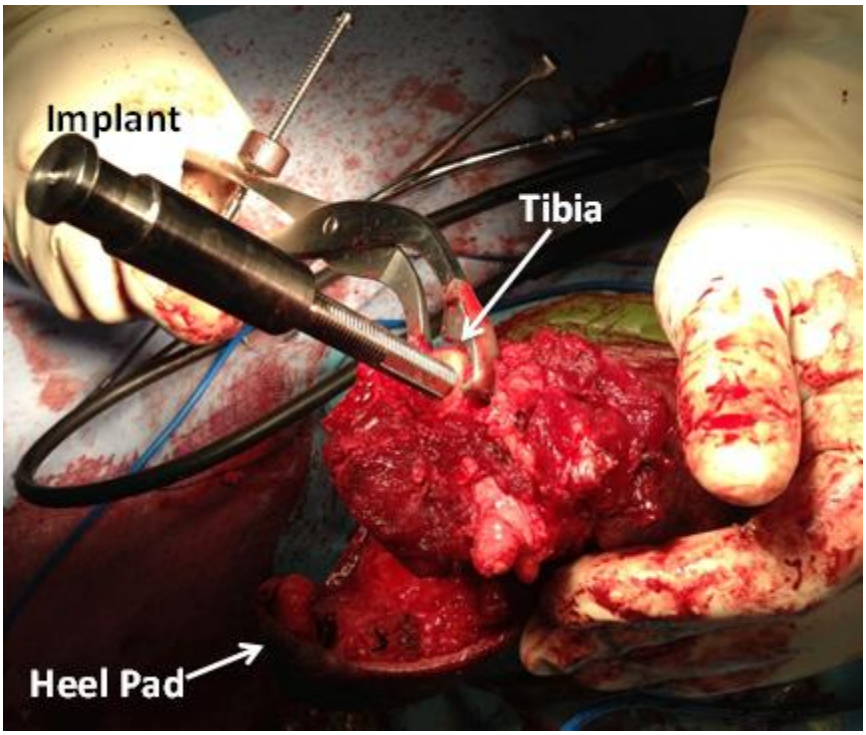

Figure 19. Initial partial insertion of implant into tibial canal.

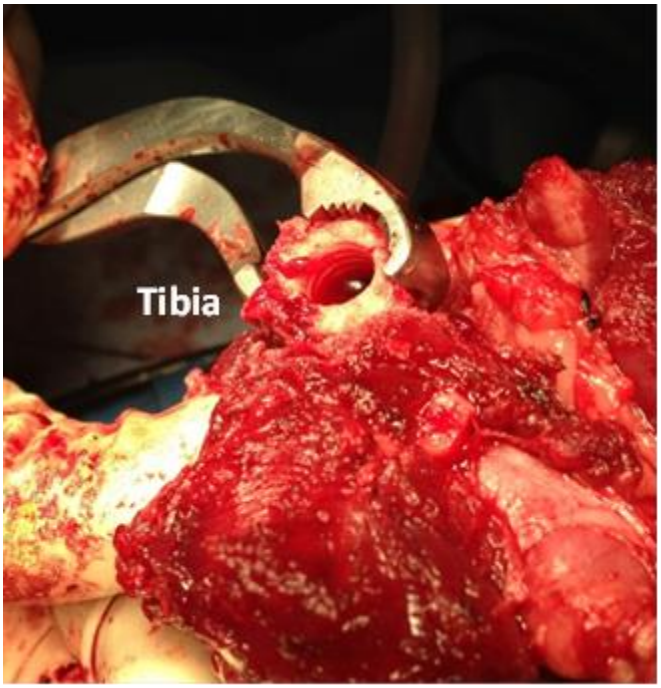

Figure 20. Initial threads in medullary canal, prior to wound closure.

Once the initial threads had been created, the implant was removed, and wound closure began. Both the anterior and posterior muscle flabs were de-bulked to allow for closure. First, the muscle layer was closed by bringing the posterior muscle flap anteriorly over the end of the remaining tibia and fibula and sutured to the anterior muscle flap. Second, the heel pad was brought anteriorly and sutured to the remaining skin anterior to the wound. Once the wound was completely closed, a K-wire was used to locate the medullary canal. Using the K-wire as a guide, a stainless steel tube sharpened at one end [Figure 21] was fed over the K-wire and used to punch a hole in the skin and muscle layer down to the level of the bone, mimicking the hole punch Aschoff uses to create a stoma in human patients.

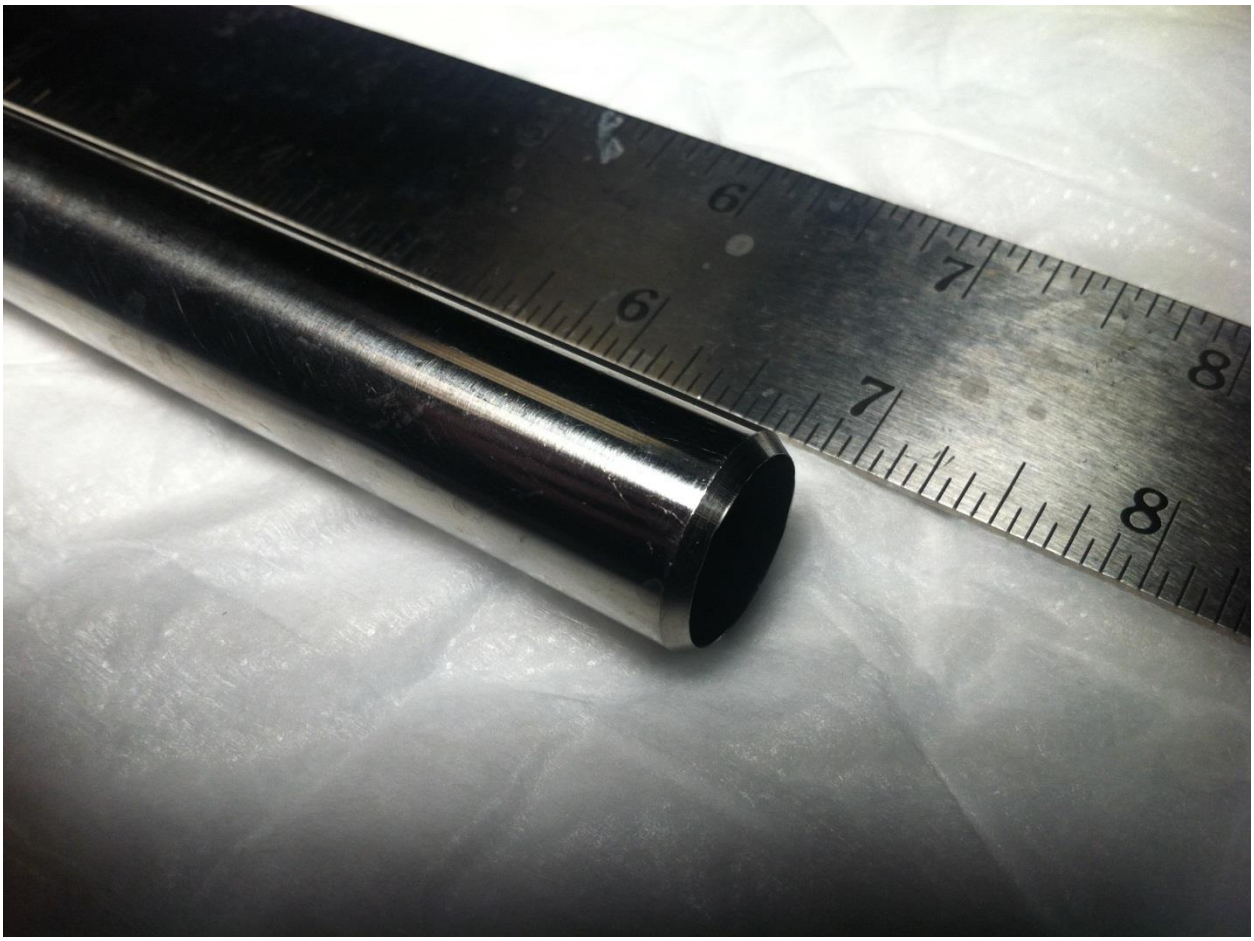

Figure 21. Surgical hole punch, sharpened at one end.

With the stoma made and the distal end of the bone visible and inset approximately 1 inch from the surface of the skin, bone cement was injected into the medullary canal. Immediately following this injection, the implant was inserted without difficulty by threading the implant into the canal. Once insertion of the implant was complete, two additional swabs were collected: one at the incision site, one at the skin-to-implant interface at the stoma site. These six swabs collected on the day of surgery were used as a baseline for the remainder of the swabs collected during the study. Sterile dressings were then applied, and x-rays were taken [Figure 22, Figure 23].

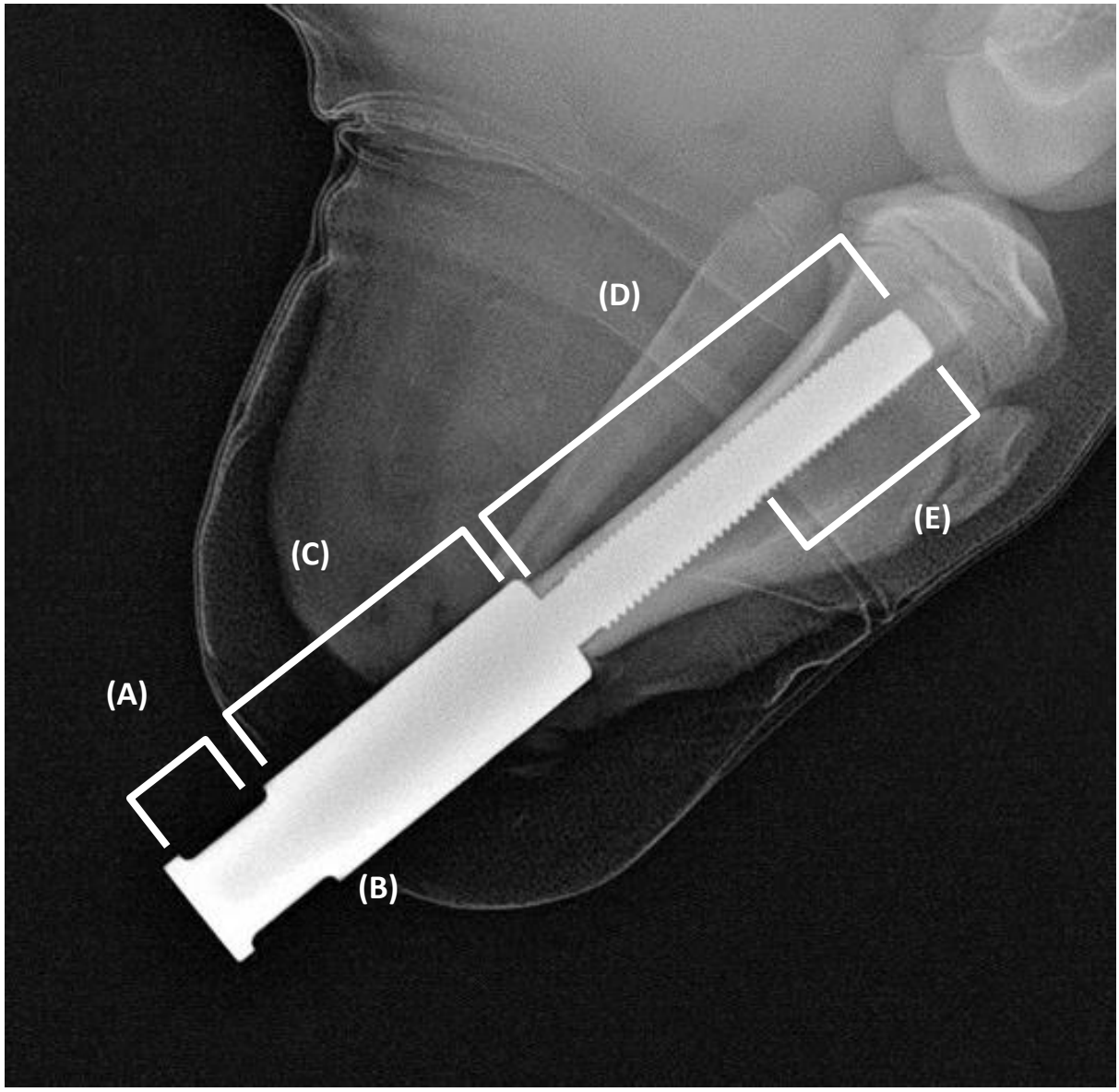

Figure 22. Post-op x-ray with implant anatomy, Pig #1. (A) prosthetic block; (B) cup and dressing; (C) skin and soft tissue; (D) bone; (E) cutting flutes.

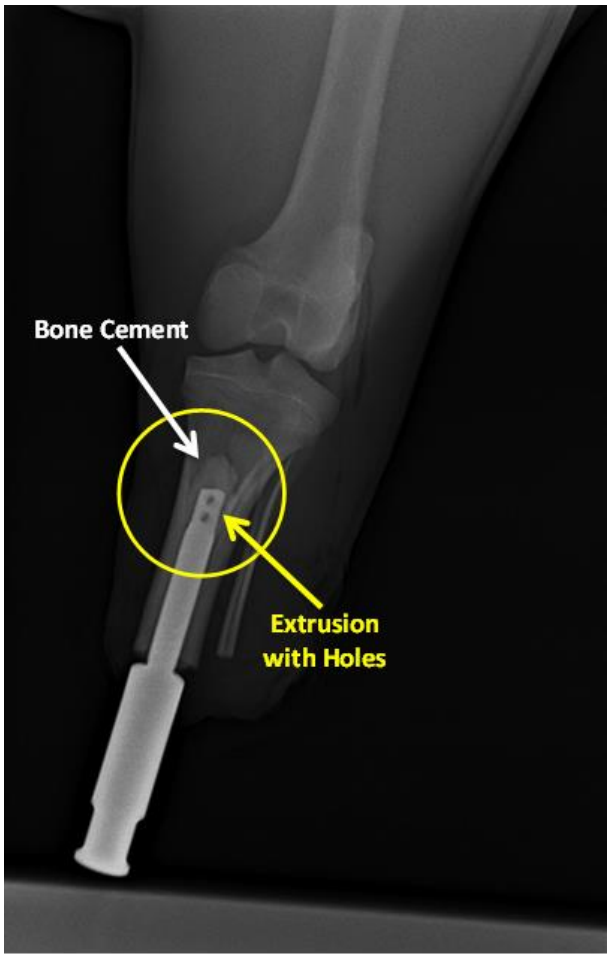

Figure 23. A/P x-ray immediately post-op, Pig #2.

Figure 22 is a post-op X-ray of Pig #1. The implant made for Pig #1 did not have the extrusion on the end with the holes that is shown in Figure 23. This extrusion was implemented in implants for Pigs #2-4 and is further discussed in 3.2 RESULTS AND DISCUSSION- SPECIFIC AIM I.

3.1.4 DRESSING THE WOUND

Triple antibiotic ointment was applied to the stoma and incision site using sterile cotton-tipped applicators. Telfa pads measuring 4”x3” were cut longitudinally in the center to provide a 1” slit. The slit allowed the Telfa pad to cover the stoma site by fitting over the exposed end

of the implant. Telfa pads were also placed over the incision site. Twelve-ply 4”x4” Gauze pads were cut in the same fashion and placed in the same manner over the implant. Gauze pads were expanded and wrapped around the incision site on the leg, covering the Telfa pads. Once all of the gauze pads were in place, the cup was placed over the distal end of the implant, essentially holding all of the dressings in position. The block [Figure 24, Figure 25] was secured in place at the notch at the distal end of the implant to prevent the cup and dressings from falling off [Figure 22]. The stump, cup, and dressings over the incision were then wrapped using Elasticon [Figure 26].

Figure 24. Angled block.

Figure 25. Straight block: (left) underside, second clamp not attached; (right) outer side, second clamp attached.

Figure 26. Stump, cup, and dressings over incision wrapped with Elasticon. Temporary block attached to keep the cup and dressings in place.

3.1.5 PROSTHESES

Prostheses development and manufacturing were primarily executed by another student in the KUMC Orthopedic Research Laboratory.[55] Two main prosthetic designs were used on all four pigs [Figure 27, Figure 28].

Figure 27. Design 1 with angled block.

Figure 28. Design 2 with straight block.

3.2 RESULTS AND DISCUSSION- SPECIFIC AIM I

The first aim of this pilot study was to perform a hind leg amputation on four pigs, modeled after an AK amputation on humans. A surgical procedure replicating a human AK amputation was developed using cadavers and applied successfully on all four pig. Each pig recovered from surgery and adjusted to walking on three legs.

During the first pig's recovery from surgery, two complications arose that allowed us to adjust our procedure for the remainder of the study:

On the first pig, we attached the initial prosthetic immediately post-op so that he would have something to replace his limb [Figure 29]. In other words, he wouldn't have to adjust to having three legs; he would just have to adjust to having a different type of leg. However, we faced a complication with recovery time due to the spinal block which led to loosening of the implant in Pig 1.

Figure 29. First pig, initial prosthetic attached immediately post-op.

During the recovery process immediately post-op, he was trying to gain his composure and tried to walk. Because of the delay in recovery from the buprenorphine spinal block, he was stumbling around putting pressure and torque on the prosthetic. At this point, there was a deep popping sound which, upon taking x-rays, we discovered was the implant breaking free of the bone cement within the medullary canal of the tibia. This was confirmed over the next couple of weeks when we observed that the implant was being slowly backed out of the bone.

The left hind leg of the first pig was the one that was amputated. When the pigs would turn left or rotate direction when they walked, they would stand on the hind leg that was in the direction they were turning (stand on their left hind leg if they were turning left) and pivot. Due to the direction of the threads, as the pig would pivot on the prosthetic, it would turn, or "unscrew" the implant from the bone. Although there was no evidence found that related this rotating implant with any gait problems,

discomfort, or wound healing, we changed the model so that the second pig and all future pigs would have their right leg amputated. If this loosening ever became a problem again, the implant would be tightened every time the pig would turn right.

These complications resulted in four changes:

1. Used morphine for spinal blocks instead of buprenorphine. Morphine has a much faster recovery time, so the remaining animals were able to regain feeling and composure more quickly post-surgery.
2. Wrapped residual limb to body as shown in Figure 30 for the first two days post-op and then attached prosthetic. This allowed the animal to acclimate to only having three legs. It also gave plenty of time for the bone cement to set before applying a load.
3. Added holes at the proximal end of the implant. The holes provided more surface area for the bone cement to become geometrically fit with the implant and better hold it in place.
4. Amputated right back leg. In case the implant ever loosened from the bone cement, the torque exerted by the back right leg would be constantly working to "screw in" the implant instead of back it out.

The remaining 3 pigs recovered much more quickly from surgery than the first pig, and none of them faced any post-operative complications. None of the other implants loosened throughout the remaining studies.

Figure 30. Pig #1 immediately post-surgery: residual limb is wrapped close to the body for protection during the animal's acclimation to limb-loss and recovery from surgery.

Chapter 4. Specific Aim II

Prevent infection while monitoring and promoting wound healing

4.1 METHODS AND MATERIALS

4.1.1 MONITORING INFECTION

Due to the high infection rate in humans, infection in the animal model was of high concern. BBL™ CultureSwab™ Collection and Transport System ("swab") was used to monitor bacteria and yeast throughout the study [Figure 31]. These swabs were collected immediately pre-op, immediately post-op, and at every dressing change throughout the study. The swabs were sent to St. Luke's Regional Laboratories in Kansas City, Missouri. At St. Luke's, a bacterial culture was performed on each swab, and categorical reports were returned to us that contained the amount (none, rare, few, moderate, many) and type of bacteria. Any levels of yeast were also included in the report. The bacterial culture results allowed me to monitor any potential signs of infection and treat them with antibiotics.

Figure 31. BBL CultureSwab collection and transport system.

4.1.2 DRESSING CHANGES

Every two to four days, the wound was cleaned and the dressings changed. We began by sedating the pig using isoflurane gas (iso) for each dressing change. This allowed us to work freely with the wound, stoma, and prosthetic without causing the animal to panic. We

completed dressing changes on one animal before starting on the next so that only one pig was sedated at any given time.

Once the pig was fully unconscious, we began unwrapping the Elasticon from the cup and leg. At every dressing change, pictures were taken of the soiled dressings, the incision site until the sutures were removed, and the stoma site. Swabs were collected at the incision site until it had healed and at the stoma site around the implant. To obtain the most accurate results of bacterial growth in the wound, the swab was rubbed against the tissue that was in direct contact with the implant inside the wound. In order to avoid contamination, no other surfaces were touched with the swab. The wounds were then cleaned using a hydrogen peroxide (H_2O_2) + water (H_2O) solution or a soap + H_2O solution. H_2O_2 + H_2O solution was chosen to guarantee prevention of any bacterial infection; soap + H_2O solution is the current method of cleaning the stoma in humans. H_2O_2 + H_2O solution was only used to clean the wounds of the first two pigs until approximately two weeks prior to sacrifice, at which time we switched to soap + H_2O. Soap + H_2O solution was used to clean the wounds on Pigs 3 and 4 for the duration of the study. (See Figure 10 and **Error! Reference source not found.** for a full timeline of the study.) Twelve-ply 4”x4” gauze pads were used to gently scrub the wound to remove any discharge, dried or otherwise. Dressings were applied in the same manner as described in 3.1.4 with a few minor variations throughout the study.

4.1.1.1 Dressing Change Variations

For the first two weeks post-op while the incision site healed, triple antibiotic ointment and Telfa pads were applied to the incision as described above in 3.1.4 . After the sutures were

removed at approximately two weeks post-op, we stopped applying ointment and Telfa pads to the incision site for the remainder of the study.

At the end of the first dressing change at two days post-op, the prosthetic was attached to the block, which was then attached to the implant. For the remainder of the study, the block-prosthetic combination was removed at the beginning of every dressing change to access the wound and attached at the end of every dressing change before the animal recovered from being sedated.

4.2 RESULTS - SPECIFIC AIM II

4.2.1 SWAB RESULTS

Two swabs were collected on each animal during preparations the day of surgery, before sterilization: one from the skin on the outside of the leg, and one from the skin on the inside of the leg. The cultures found and their reported amount classifications are shown in Table 2. In order to verify that our sterilization methods for surgery were successful and that the wound was not contaminated during surgery, four additional swabs were taken in the same locations as before sterilization. Each post-sterilization and immediate post-operation swab reported no growth at up to five days. The only difference in type of bacterium found pre- vs. post-op is that klebsiella pneumonia (KP) and acinetobacter baumannii (AB) were found pre-op, and stenotrophomonas maltophilia (SM) and bacillus species were found post-op.

Table 2. Cultures Found Pre-Surgery, Separated by Pig

Bacteria Found Per Pig Pre-Op		
Bacteria	**Outside Leg Swab**	**Inside Leg Swab**
Pig #1		
Yeast		Few
Mixed Gram Negative Rods	Many	Many
Staphylococcus Species, Coagulase Negative	Moderate	
Pig #2		
Mixed Gram Negative Rods	Moderate	Moderate
Pseudomonas Aeruginosa	Moderate	Moderate
Mixed Gram Positive Cocci	Moderate	Moderate
Pig #3		
Klebsiella Pneumoniae	Many	Many
Acinetobacter Baumannii	Many	Many
Enterococcus Species	Few	Moderate
Pseudomonas Putida	Moderate	
Pig #4		
Acinetobacter Baumannii	Many	
Gram Negative Rods		Few
Mixed Gram Positive Flora	Many	Few

Post-op bacterial culture reports from the four animals combined contained the following at least once throughout the study: Yeast, Enterococcus Species, Mixed Gram Negative Rods, Staphylococcus species (coagulase negative), Pseudomonas Aeruginosa, Bacillus Species, Pseudomonas Putida, Stenotrophomonas Maltophilia, and Gram Positive Cocci. A total of 41 swabs were collected post-op during the time when H_2O_2 was being used to clean the wound on the first two animals, and 73 swabs were collected over all 4 animals during the time soap was being used to clean the wound for a total of 114 swabs collected. Swabs collected that returned “No Growth” after three days are classified under “Nothing Found” and totaled 3

for the time H_2O_2 was used and 16 otherwise. Only yeast and 3 types of bacteria were found during the time H_2O_2 was used, as compared to yeast and 8 different types of bacteria present when only soap and water were used.

Using a classification system for amount reported where none = 0, rare = 1, few = 2, moderate = 3, and many = 4, the amount of each bacteria reported was added together for both methods of wound cleaning. More than one bacterium could be reported per swab collected. The frequency is the number of times that bacteria was reported and is shown in Table 3 along with the total amounts of each. Figure 32 and Figure 33 show the percentage of the total amount and frequency of each bacterium for both methods of cleaning the wound.

Table 3. Combined Bacterial Culture Report for All Pigs

Combined Bacterial Culture Report - All Pigs				
	Frequency, with H2O2	**Frequency, without H2O2**	**Total Amount, with H2O2**	**Total Amount, without H2O2**
Yeast	28	21	90	67
Enterococcus Species	8	8	25	26
Gram Negative Rods	3	31	6	100
Staphylococcus species, coagulase negative	2	4	6	12
Nothing found	3	16		
Pseudomonas Aeruginosa		19		58
Bacillus Species		1		3
Pseudomonas Putida		1		2
Stenotrophomonas Maltophilia		1		3
Gram Positive Cocci		1		2
Total Sum	**44**	**103**	**127**	**273**

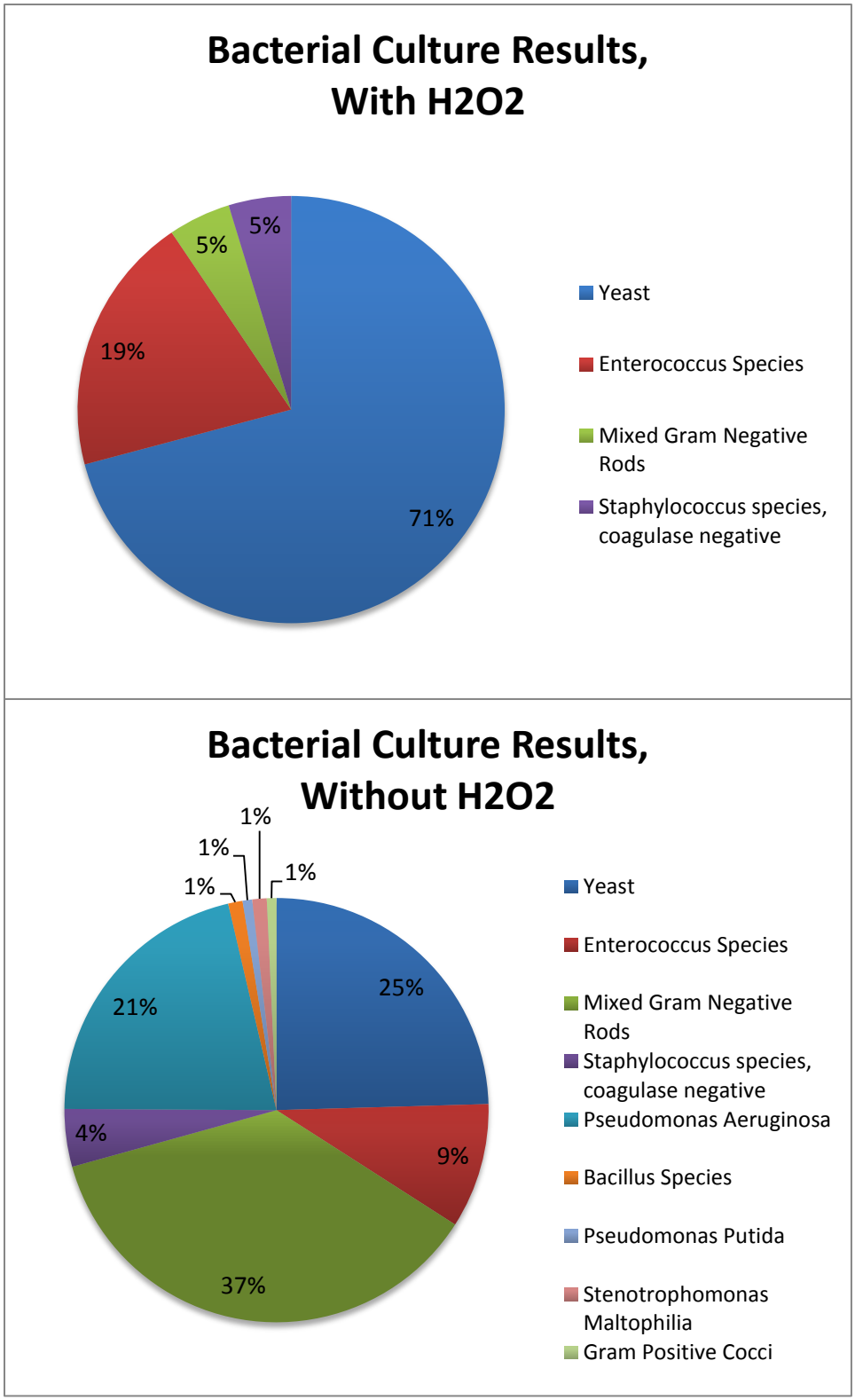

Figure 32. Bacterial culture results - comparing with hydrogen peroxide vs. without hydrogen peroxide, post-surgery, all animals.

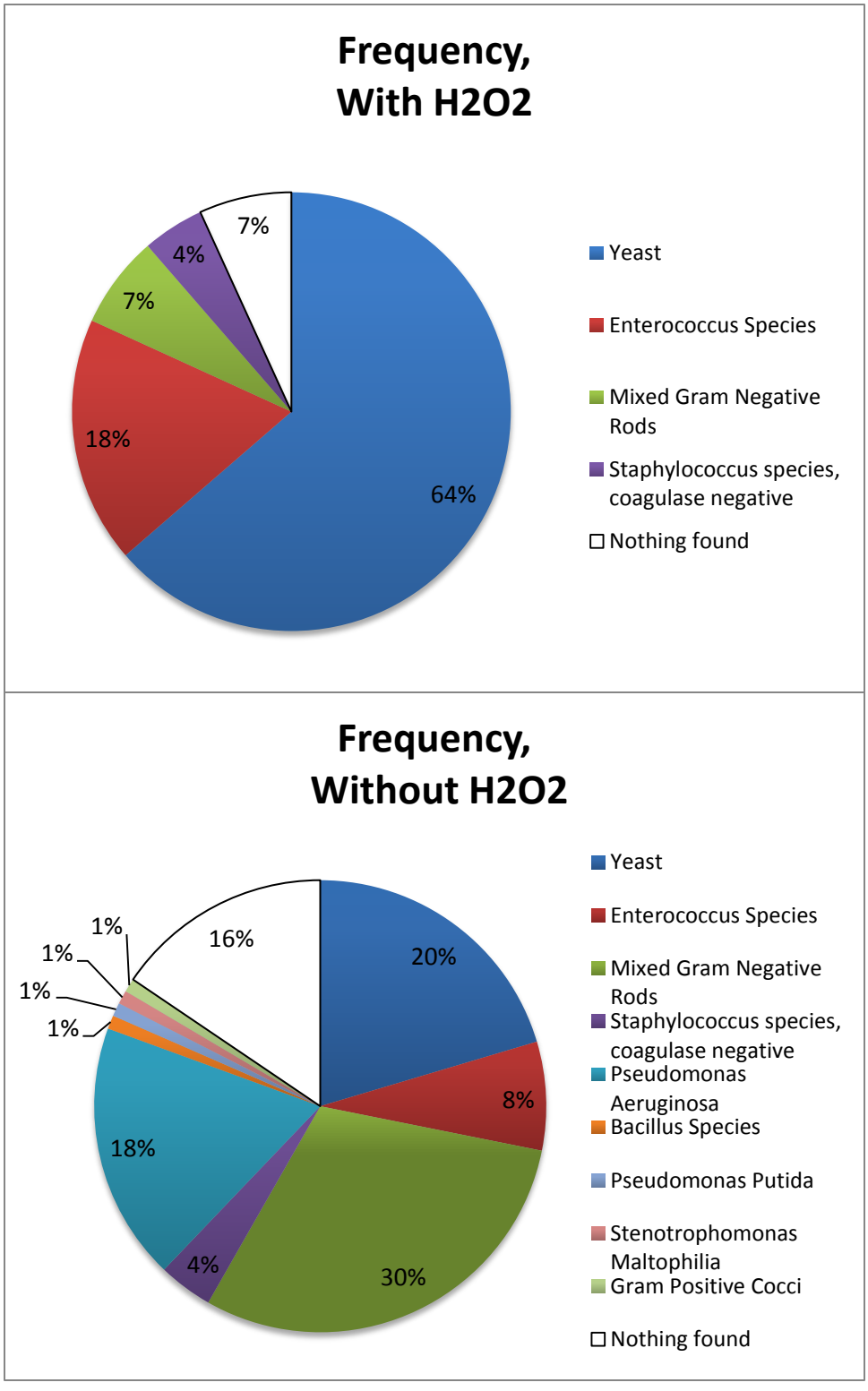

Figure 33. Bacteria culture report - frequency of bacteria and yeast found throughout the study: post-surgery, all animals.

While yeast was found during both methods of cleaning, 5 bacteria were found only while cleaning with soap and water: Pseudomonas Aeruginosa, Bacillus Species, Pseudomonas Putida, Stenotrophomonas Maltophilia, and Gram Positive Cocci. There were almost six times as many mixed gram negative rods found while using only soap and water as were found using H_2O_2. For a direct comparison between methods of cleaning, Table 4 shows a comparison for only the cultures found while using H_2O_2. Staphylococcus species was found twice as often while using soap and water, but only makes a .5% difference when compared to the frequency of all bacteria listed in Table 4.

Table 4. Direct Comparison of Bacterial Culture Results

Bacterial Culture Report - All Pigs, Direct Comparison				
	Frequency, with H2O2	**Frequency, without H2O2**	**Total Amount, with H2O2**	**Total Amount, without H2O2**
Yeast	28	21	90	67
Enterococcus Species	8	8	25	26
Gram Negative Rods	3	31	6	100
Staphylococcus species, coagulase negative	2	4	6	12
Nothing found	3	16		
Total Sum	**44**	**80**	**127**	**205**
Percentages				
Yeast	63.6	26.3	70.9	32.7
Enterococcus Species	18.2	10.0	19.7	12.7
Mixed Gram Negative Rods	6.8	38.8	4.7	48.8
Staphylococcus species, coagulase negative	4.5	5.0	4.7	5.9
Nothing found	6.8	20.0		

Table 5 shows the frequency of cultures found in each pig. The average amount found for each culture while only using soap and water was a rating of 2 (classification of 'few') or greater. While there were four cultures found in Pig #1 that were only present after stopping H_2O_2, all cultures that were present in Pig #2 while using H_2O_2 were also present while using soap+ H_2O solution. There was no direct relationship between type or classification of bacterium and day post-surgery collected.

Table 5. Type, Frequency, and Average Amount of Bacteria Found, Separated by Pig

Frequency of Bacteria Found - Per Pig				
Bacteria	**Frequency Found, With H_2O_2**	**Average Amount, With H_2O_2**	**Frequency Found, Without H_2O_2**	**Average Amount, Without H_2O_2**
Pig #1 (8 Weeks - 56 Days) 41 Days with H_2O_2, 15 Days without H_2O_2				
Yeast	18	3.2	8	3.4
Mixed gram negative rods	2	1.5		
Staphylococcus species, coagulase negative	1	3		
Bacillus Species*			1	3
Pseudomonas Putida*			1	2
Enterococcus Species*			3	3.3
Pseudomonas Aeruginosa*		3.5	2	3.5
*Found only after stopping H2O2				
Pig #2 (5 Weeks - 35 Days) 20 Days with H_2O_2, 15 Days without H_2O_2				
Yeast	10	3.2	8	3.1
Mixed gram negative rods	1	3	1	3
Staphylococcus species, coagulase negative	4	3	4	3
Enterococcus Species	8	3.1	5	3.2
Pig #3 (11 Weeks - 78 Days)				
Yeast			4	3.3
Mixed gram negative rods			15	3.3
Stenotrophomonas Maltophilia			1	3.0
Pseudomonas Aeruginosa			6	2.8
Gram Positive Cocci			1	2.0
Pig #4 (10 Weeks - 70 Days)				
Yeast			1	2.0
Mixed gram negative rods			16	3.0
Pseudomonas Aeruginosa			11	3.1

4.2.2 WOUND HEALING RESULTS

Wound healing analysis was primarily performed by another student in the KUMC Orthopedic Research Lab[55] and is included in this thesis to demonstrate evidence that the wound was healing but did not create a definitive seal at the skin-to-implant interface.

4.2.2.1 Visual Inspection

Pictures of the wound were taken at every dressing change to visually monitor wound healing progression throughout the study. Figure 34 shows a progression of wound healing of the stoma from Day 0 (top left) taken immediately post-op, to Day 78 (bottom right) taken the day of sacrifice of Pig #3. In all four pigs, the surgical incision (SI) shown in Figure 34 (d) was healed around day seven, and the sutures were removed around day fourteen. Dried exudate is shown in Figure 34 (c). Figure 34 (e) shows a thin line of granulation tissue (GT). More GT was present on Day 40, and the GT visible on Day 59 is bright red with a texture on the surface of the tissue as shown in Figure 34 (g). Between Days 59 and 78, the color of the wound changed from red to brown, and the texture changed from bumpy to smooth.

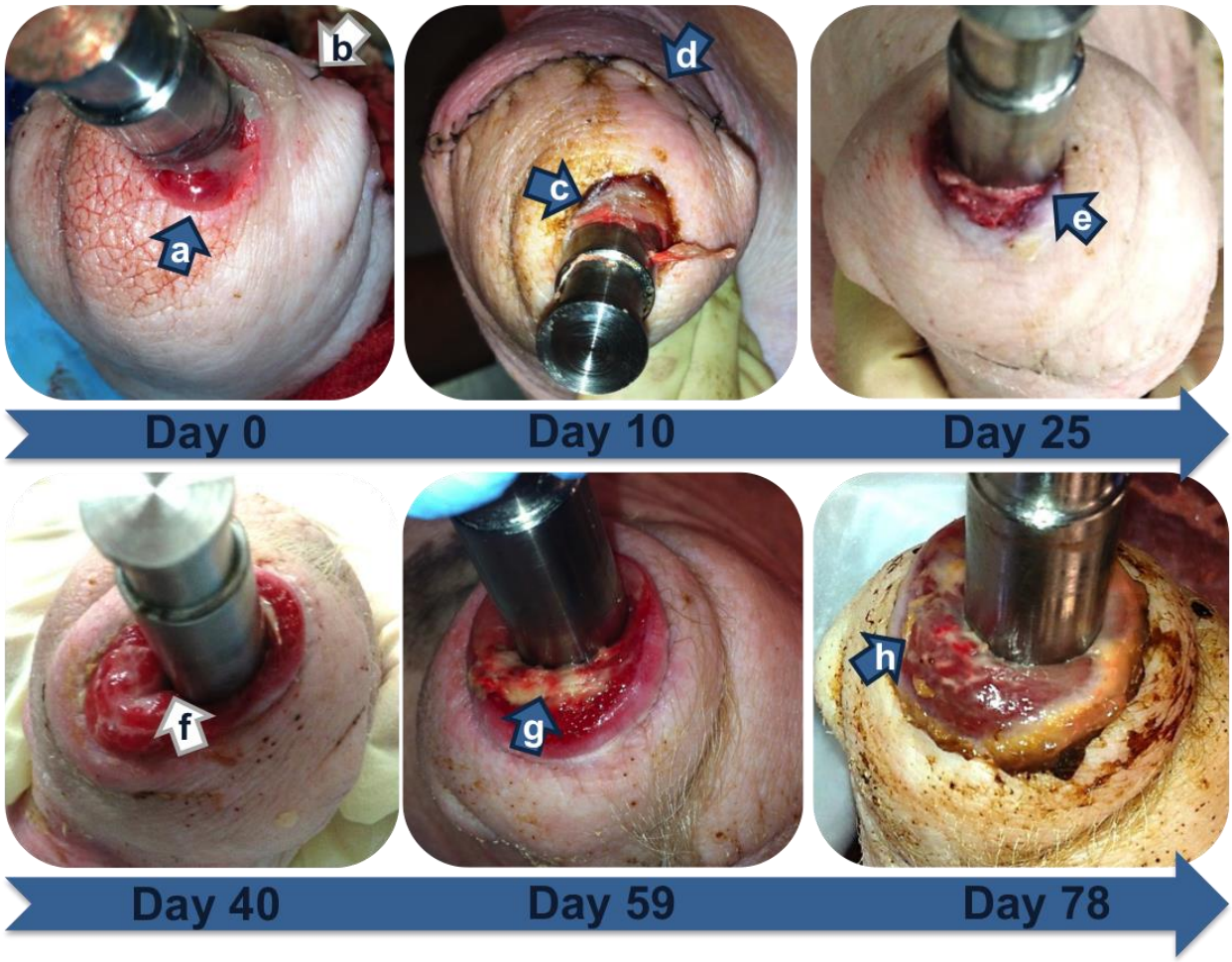

Figure 34. Wound healing progression in Pig #3: (a) stoma, (b) surgical incision (SI), (c) dried exudate, (d) SI healed at approximately one week, (e) evidence of remodeling, (f) granulation tissue (GT), (g) more pronounced GT, or "proud flesh", (h) epithelialization occurring.

4.2.2.2 Histology

Necropsy was performed immediately post-sacrifice. Tissue samples were collected from the stoma site and surrounding tissue. Marsha Danley, the UPA Director of Immunohistochemistry in the Department of Pathology and Laboratory Medicine at the University of Kansas Medical Center, cut and stained the tissue samples and made slides. Histological analysis was performed by another student in the KUMC Orthopedic Research Lab.[55] The results determined by this student can be found in A6. Histology.

4.3 DISCUSSION – SPECIFIC AIM II

Large amounts of yeast were found in both animals while using H_2O_2. While the H_2O_2 successfully killed any potentially threatening bacteria, it also killed any healthy cultures needed for wound healing, and consequently allowed the yeast to thrive. According to the LAR veterinarian involved with medical care of the animals, all culture types found could be classified as "normal" for the skin and environment of pigs and didn't directly mean the wound was infected. There were no clinical signs of infection (smell, excessive or unusual discharge, inflammation, or spike in temperature) in any animal.

The dried exudate shown in Day 10 (c) of Figure 34 (top middle) was considered normal discharge. There was evidence of remodeling as shown in the picture from Day 25 (e) (top right) by the thin line of GT formation. This GT was more present on Day 40 (f) (bottom left) and even further pronounced on Day 59 (f) (bottom middle). Healthy GT is characterized by many blood vessels that form on the surfaces of a wound during healing and results in the skin appearing bright red.[56, 57] Excessive granulation tissue, or "proud flesh", was present on Day 59 (bottom middle) and indicated by small bumps on the surface of the wound. This proud flesh has been reported as painless when healthy[58] and is not expected to have affected gait. On Day 78 (bottom right), the GT had been replaced by the beginning formation of new epithelial tissue.

Enterococcus species, mixed gram negative rods, staphylococcus species, bacillus species, and gram positive cocci are all categories or types and do not represent any one specific bacterium.

Chapter 5. Specific Aim III

Collect and analyze both visual and numerical data on kinematic and kinetic gait parameters both pre-operation (pre-op) and post-operation (post-op) to determine how well the animals adapted to the amputation, as well as the potential impact the amputation had on the other joints.

5.1 GAIT STUDY: THE SETUP

Figure 35 shows how the data collection equipment was set up during the gait studies. The equipment was located no more than six meters and no less than four meters from the center of the force plate during data collection.

5.1.1 MOTION CAPTURE SYSTEM

Dartfish Software 6 ProSuite 6.0 build 1130 (Dartfish, Alpharetta, Georgia) ("Dartfish") was used to record and analyze the video captured by the video camera at 24 frames per second during the experiment. Dartfish had previously been installed on the computer shown in Figure 35 B. The video camera was positioned perpendicular to the x-axis of the force plate and centered with the origin. As a limitation of the study, the force plate and motion capture system were not linked in any way.

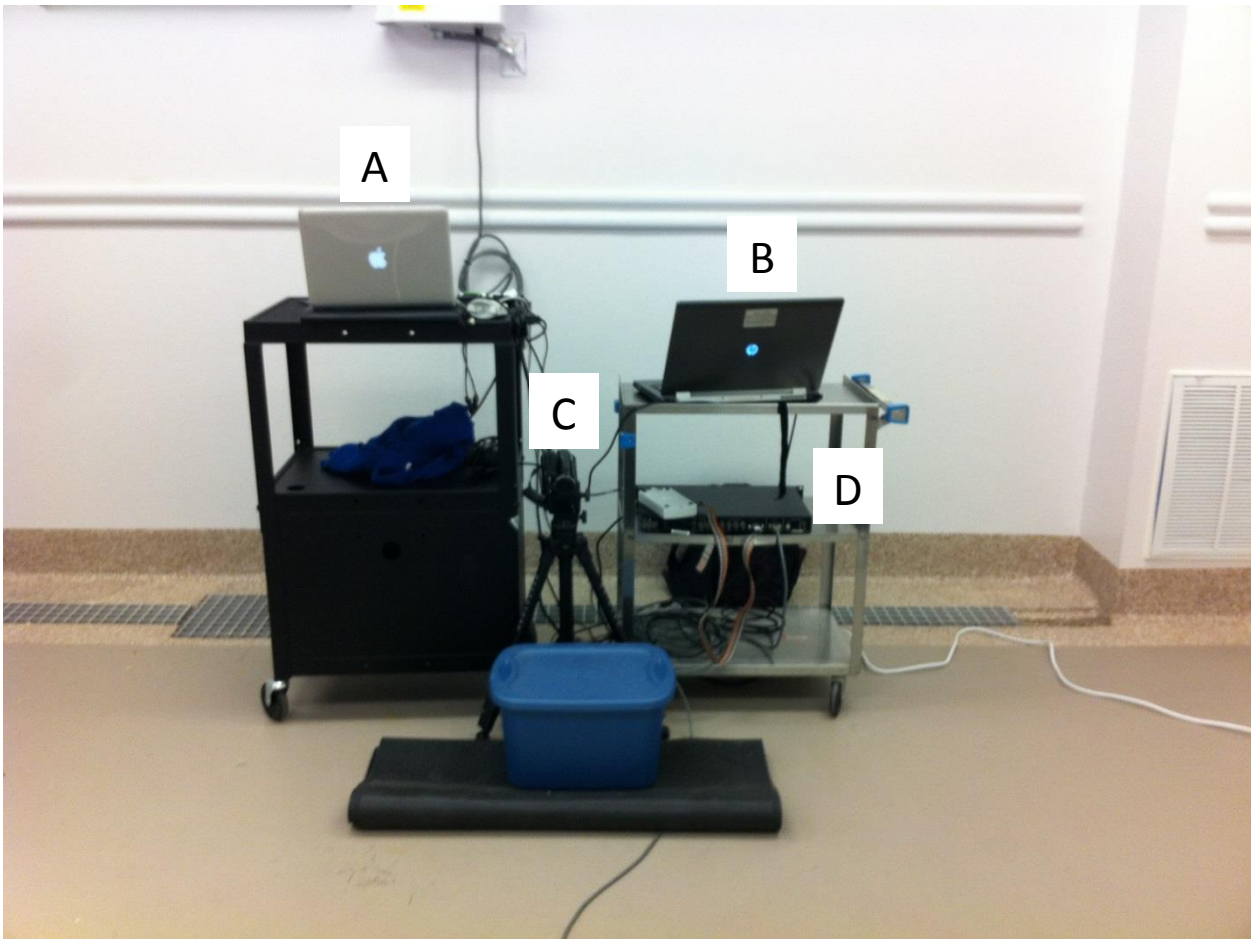

Figure 35. Gait study, data collection equipment set-up: A) computer for recording numerical force plate data using LabVIEW; B) Dartfish computer for recording and analyzing video gait; C) camera for capturing video; D) 6-channel Bertec amplifier AM6900.

5.1.2 FORCE PLATE

A four-load-cell Bertec force plate (Bertec Corporation, Columbia, OH; model number 4060-NC) was used in combination with a Bertec six-channel digital to analog amplifier for the gait study. Standard Bertec force plate coordinates can be found in Figure 36. Force plate data were collected at a sampling rate of 1,000 Hz using LabVIEW software 6.1 (National Instruments Corporation, Austin, TX).

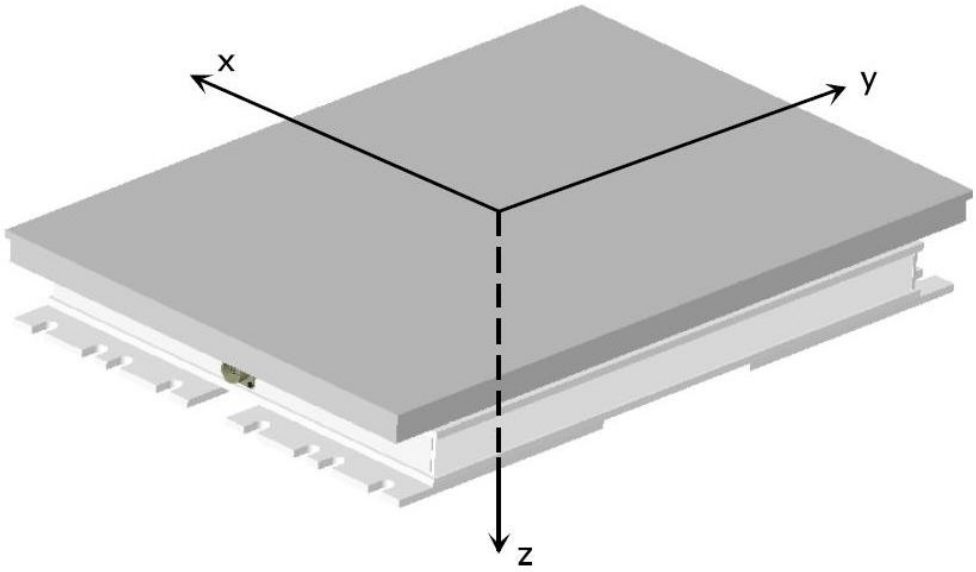

Figure 36. Standard Bertec Force Plate Coordinate System: the origin is on the top surface, and at the center of the plate. Positive y-direction is opposite to the connector end; x-axis is to the left when looking in the y-axis; and the z-axis is defined downwards by the right hand rule.

Image reproduced from Bertec Force Plate Manual (2005) [4]

5.1.3 PLATFORM

A raised platform was constructed to house the force plate for the experiment. The platform consisted of three sections and two ramps [Figure 37]. The dimensions can be found in A7. Platform Drawing. The frame was constructed out of 2”x4”s and screws, and plywood sheets were screwed into the frame. It was then painted white to seal the wood as per LAR requirements. Black carpet was tacked down to the top of the platform to provide traction [Figure 37]. The force plate was placed in a cutout in the middle of the platform and covered with double-sided tape and carpet to make it indistinguishable to the animals [Figure 38 and Figure 39].

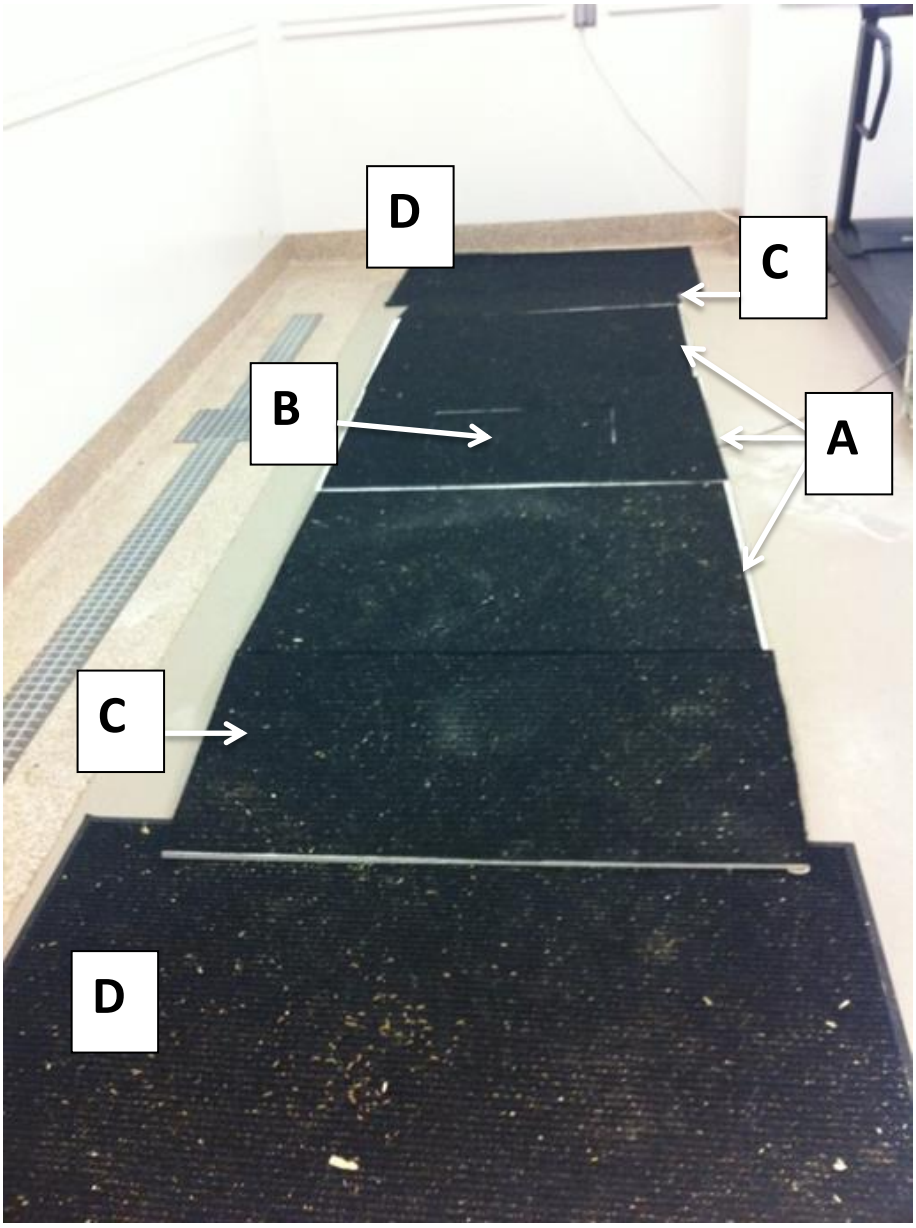

Figure 37. Platform for Gait Study. A) 3 Platform pieces, B) Force Plate, C) Ramps, D) Extra carpet on the floor to provide additional traction.

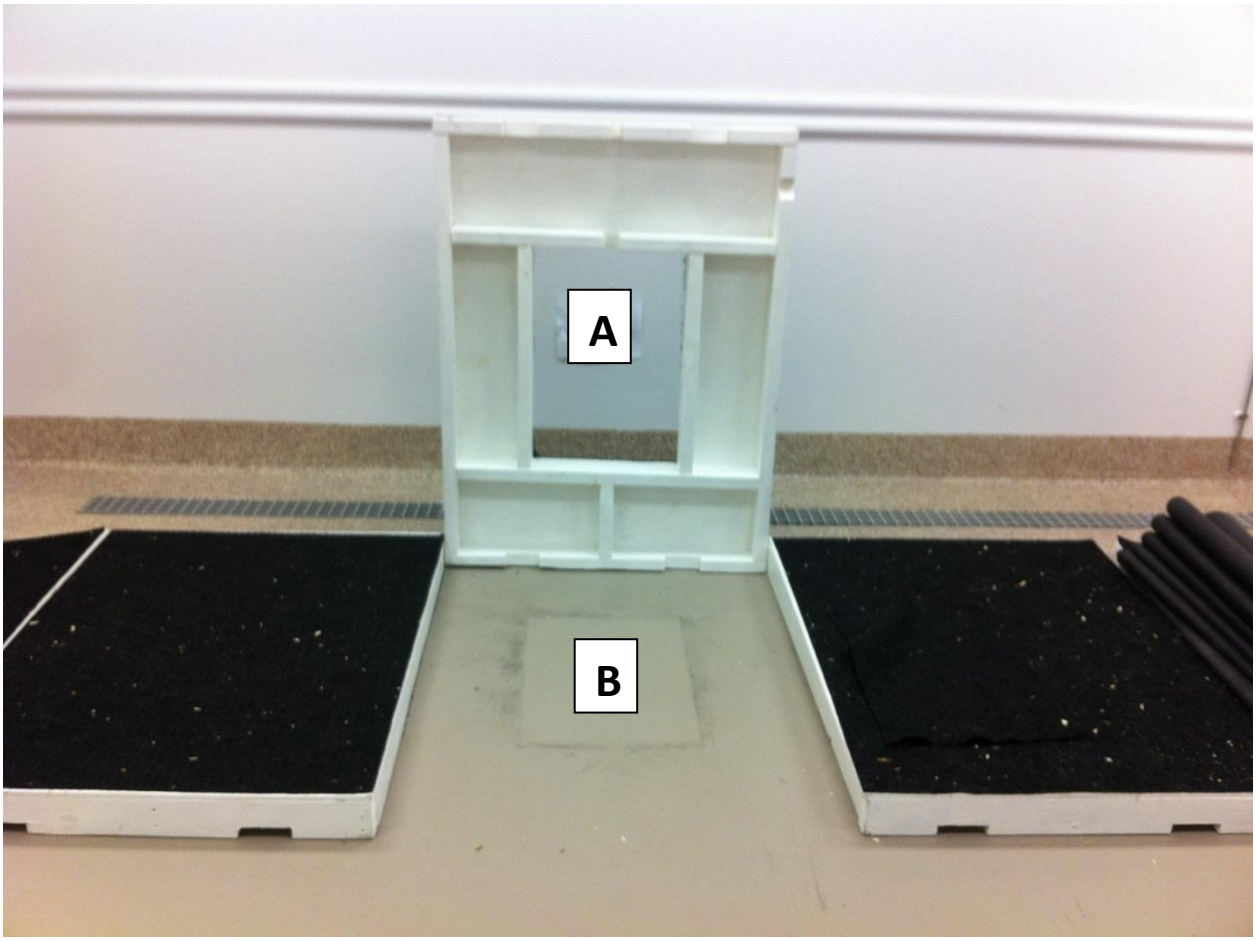

Figure 38. Cutout in platform (A) to show where the force plate sits (B). The underside of the middle section of the platform is shown.

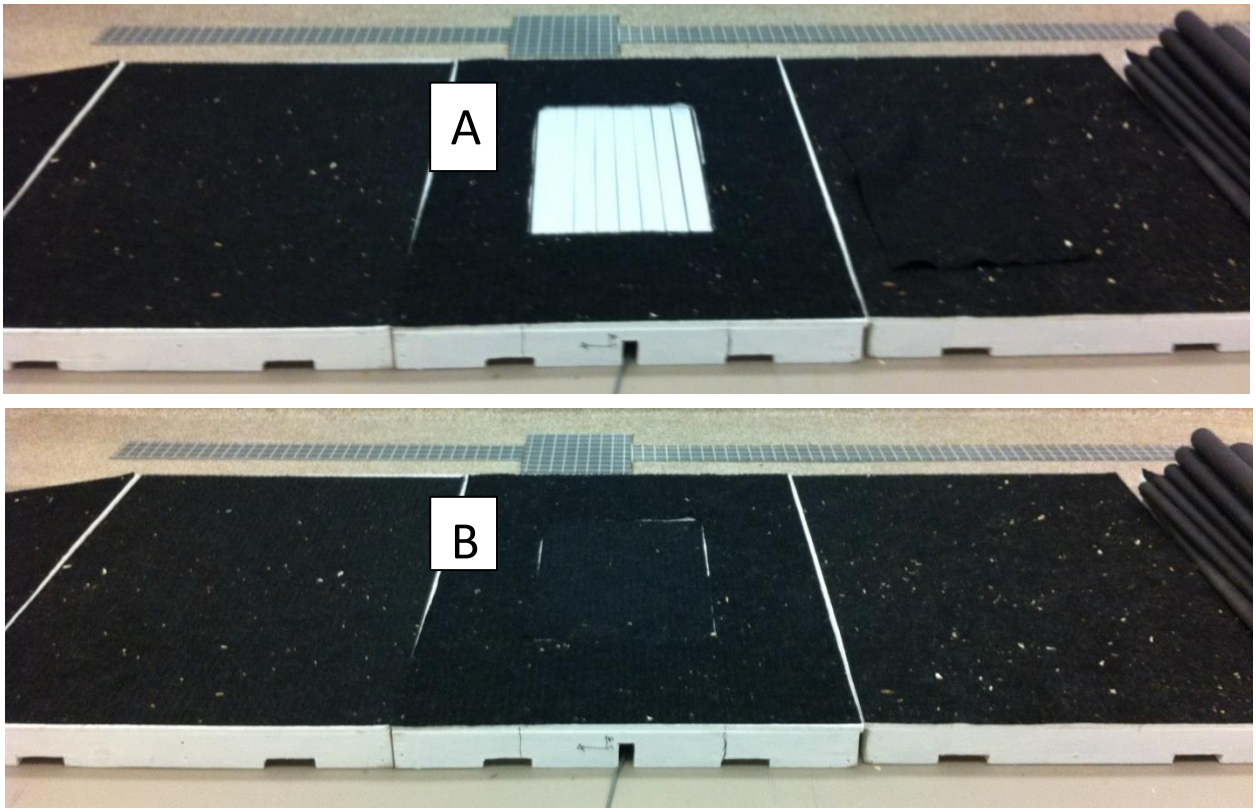

Figure 39. Force plate in platform, before (A) and after (B) carpet covering was applied. Before the carpet covering was applied, it was covered with double-sided carpet tape.

5.2 GAIT STUDY: THE EXPERIMENT

Data were collected on only one animal at a time. After attaching the harness and leash to the pig, he was led out of his pen to an adjacent room where data were collected on his gait. Using food items as treats, the pig was led across the platform from one end to another so that he would walk with at least one hoof on the force plate at a time. The equipment operator (EO) stood at the equipment station [Figure 35] and operated both computers while making note of what each trial was named, how the animal ambulated, and which direction the animal traveled for each trial. Each time the pig walked across the platform, EO recorded the data as a single trial using LabVIEW at a sample rate of 1,000 Hz. This process was repeated at various

time points throughout the study on each pig (see Figure 10 and **Error! Reference source not found.** for the exact timing of gait collection on each pig).

5.3 DATA ANALYSIS

Due to the variation in study duration for each animal and subsequent data collection times across each pig [Figure 10 and **Error! Reference source not found.**], the data were separated into quarters, where:

- PRE is a combination of all data collected pre-op,
- Q1 is a combination of all data collected weeks 1-4 post-op,
- Q2 is a combination of all data collected weeks 5-8, and
- Q3 is a combination of all data collected weeks 9-11.

Each quarter contained at least one data collection from all 4 pigs except for Q3, which contained data collected from only Pigs 3 and 4. The data were broken up this way to correlate more closely with the standard timeframe of clinical post-op visits in humans.

Pig #1 had a hind left leg amputation, and Pigs #2, #3, and #4 had a hind right leg amputation. For an equivalent analysis comparison between legs, all legs and their data were separated into one of four categories:

- Amputated side, front leg (AFL)
- Amputated back leg (ABL)
- Intact side, front leg (IFL)
- Intact back leg (IBL)

5.3.1 DARTFISH VIDEO ANALYSIS

Each video was taken during the entire experiment for each pig (1 experiment with many force plate trials = 1 video). In Dartfish, distances measured are only precise in a plane perpendicular to the camera.[59] This plane must first be calibrated by defining a reference distance.[59] Using the distance function within the software, a reference distance of 1 meter was defined by using a known distance on the platform of 1 meter. Due to geometric optics, an object appears smaller the farther away it is; similarly, the same object appears larger the closer it is. The reference length was affected by this phenomenon. This reference length varied depending on the plane in which the animal crossed the force plate: closer to the camera, the reference length was elongated; farther from the camera, the reference length was shortened; all the while maintaining a reference distance of 1 meter.

5.3.1.1 Stride Length

Using the distance function, the stride length of both hind legs was measured.

1. A marker was placed at the point of contact between the hoof and the ground.
2. The video was fast-forwarded until the same hoof came in contact with the ground again.
3. Another marker was placed in the same spot on the hoof at the new point of contact between the hoof and the ground.
4. The distance between the markers was then measured and a note was made of the distance between the markers. This was the **stride length** for that trial.

This process was repeated for each hind leg at every instance of steady state ambulation.

5.3.1.2 Gait Cycle

Each video was stamped with the length of the video accurate to 1/1000 seconds. Using this time stamp, the duration of gait cycle (GC) was calculated in seconds as well as the support time and swing time each instance the animal was recorded having steady-state ambulation.

1. Moving through the video frame by frame, the time at which the hoof first came in contact with the ground was recorded as the **Support Start Time**.
2. Next, the time at which the hoof first showed no visible contact with the ground was recorded as **Support End Time**.
3. The **Support End Time** was also the **Swing Start Time**.*
4. Finally, the time at which the hoof came in contact with the ground again was recorded as the **Swing End Time**.
5. The **Gait Start Time** was defined as either the **Support Start Time** or the **Swing Start Time**, whichever came first; the **Gait End Time** was defined as either the **Swing End Time** or the **Support End Time**, respectively.

*If the support cycle were not visible prior to the swing cycle, the swing cycle would be recorded first. In which case, the Swing End Time would also be the Support Start Time, the Swing Start Time would be the Gait Start time, and the Support End Time would be the Gait End Time.

The **Duration of GC** (seconds) was found by subtracting the **Gait Start Time** from the **Gait End Time**. The **Support Cycle** was taken as a percent of GC (**%GC**) by subtracting the **Support Start Time** from the **Support End Time** and dividing by the **Duration of GC**. The same was done with the **Swing Cycle** as a **%GC**.

5.3.1.3 Speed

Velocity in meters/second (m/s) was calculated by dividing the **Stride Length** in meters by the **Duration of GC** in seconds. The results were grouped and analyzed by velocity in .25 m/s increments:

- S1: 0.500-0.744 m/s
- S2: 0.750-0.999 m/s
- S3: 1.000-1.244 m/s
- S4: 1.250-1.499 m/s
- S5: 1.500-1.744 m/s
- S6: 1.750-1.999 m/s
- S7: 2.000+ m/s.

The means and standard deviations of each variable were calculated for each velocity group on each day for each animal.

5.3.1.4 Prosthetic Angle

To show evidence of a lack of extension post-surgery, the **Max** and **Min Prosthetic Angle** were calculated in all 4 pigs and were then used to calculate the **Prosthetic Angle Range** (**Max - Min**). The prosthetic angle was found by measuring the angle the implant (essentially an extension of the tibia) made with the horizontal (the ground) as shown in Figure 40.

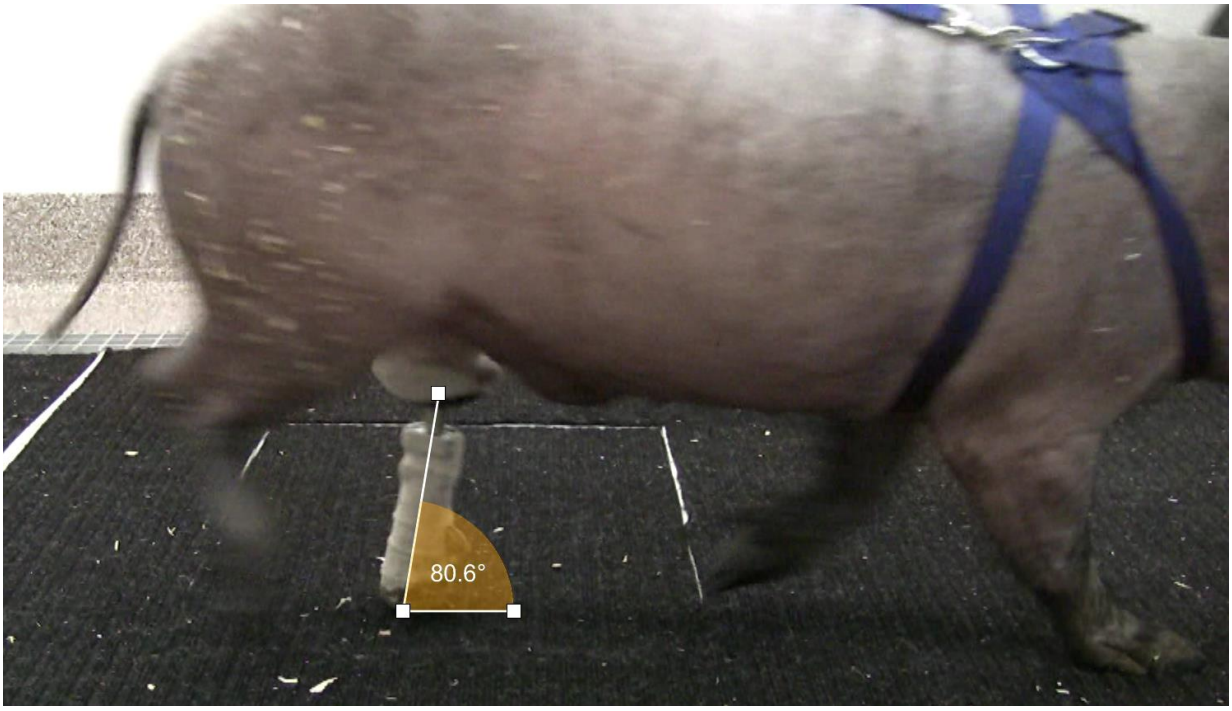

Figure 40. Dartfish snapshot – max prosthetic angle measurement with the horizon. Pig #1 day 55 post-op.

5.3.1.5 Example Gait

For a visual comparison of gait on the amputated hind leg, X and Y location data points of different anatomical landmarks pre- and post-op were recorded and plotted. These anatomical landmarks pre-op were [see Figure 41]:

A. Hip joint

B. Knee joint

C. Posterior end of calcaneus

D. Flexion point of proximal metatarsals

E. Flexion point of distal-most metatarsals

F. Most distal point of hoof

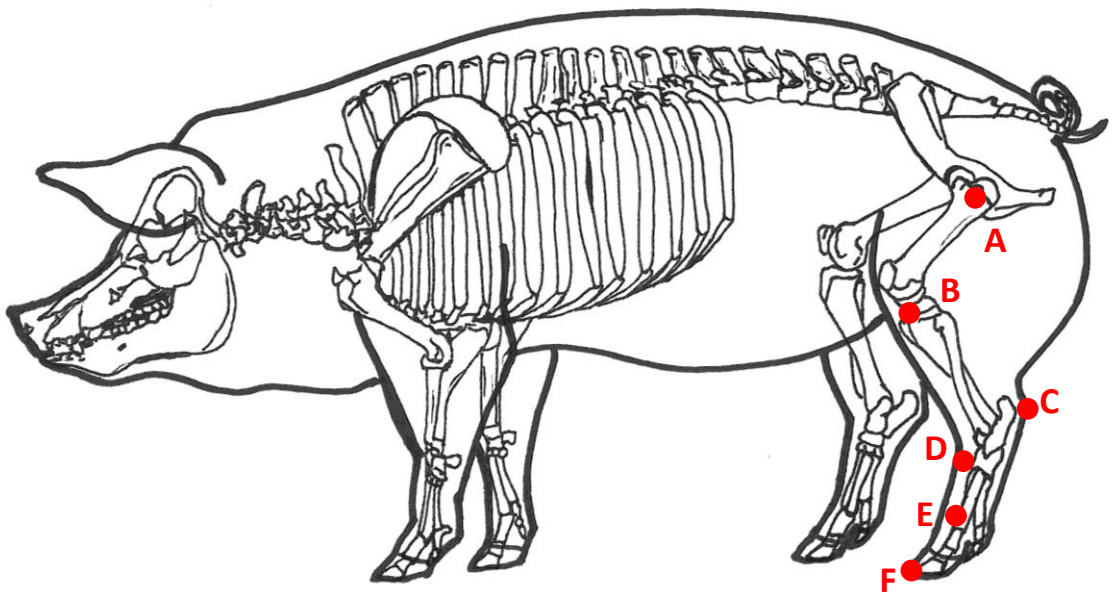

Figure 41. Pig anatomy with approximate marker locations – pre-op only.

These approximate locations post-op were [see Figure 42]:

A. Hip joint

B. Knee joint

C. Most proximal location of implant

D. Bottom of prosthetic

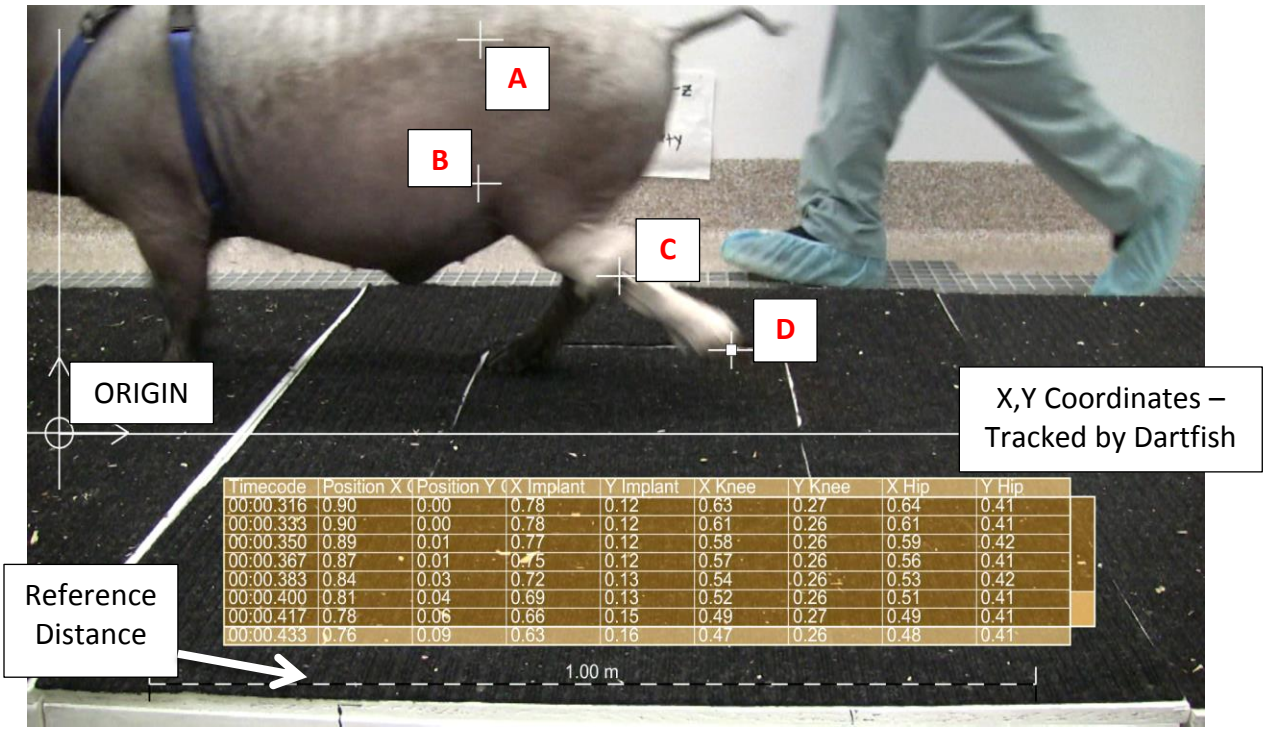

Figure 42. Dartfish snapshot - collecting (x,y) coordinates of anatomical landmarks for visual comparison of gait.

As a requirement of IACUC that no markers be placed on the pigs, the knee and hip joint locations are based solely on video documentation and anatomical landmarks.

5.3.2 FORCE PLATE

All force plate data were analyzed using MATLAB (Mathworks, Natick, MA) and Excel 2010 (Microsoft, Redmond, Washington).

The output from the force plate consisted of 7 channels:

1. Time
2. Force in X (FX)
3. Force in Y (FY)
4. Force in Z (FZ)

5. Moment about X (MX)
6. Moment about Y (MY)
7. Moment about Z (MZ)

Due to the large amount of noise in the data, the data were filtered using a moving average filter. Figure 43 is an example of one trial after filtering. Trials were excluded where there was a substantial overlap in stance between two or more limbs [Figure 44] or where steady-state gait was not achieved. For each trial where the animal was walking in the –X direction (to the right) as indicated by notes collected each day of the gait study, all FX, FY, MX, and MY data were multiplied by -1. This normalized each of the forces in x and y and the moments about x and y to be analyzed as if the animal had been walking in same direction each trial.

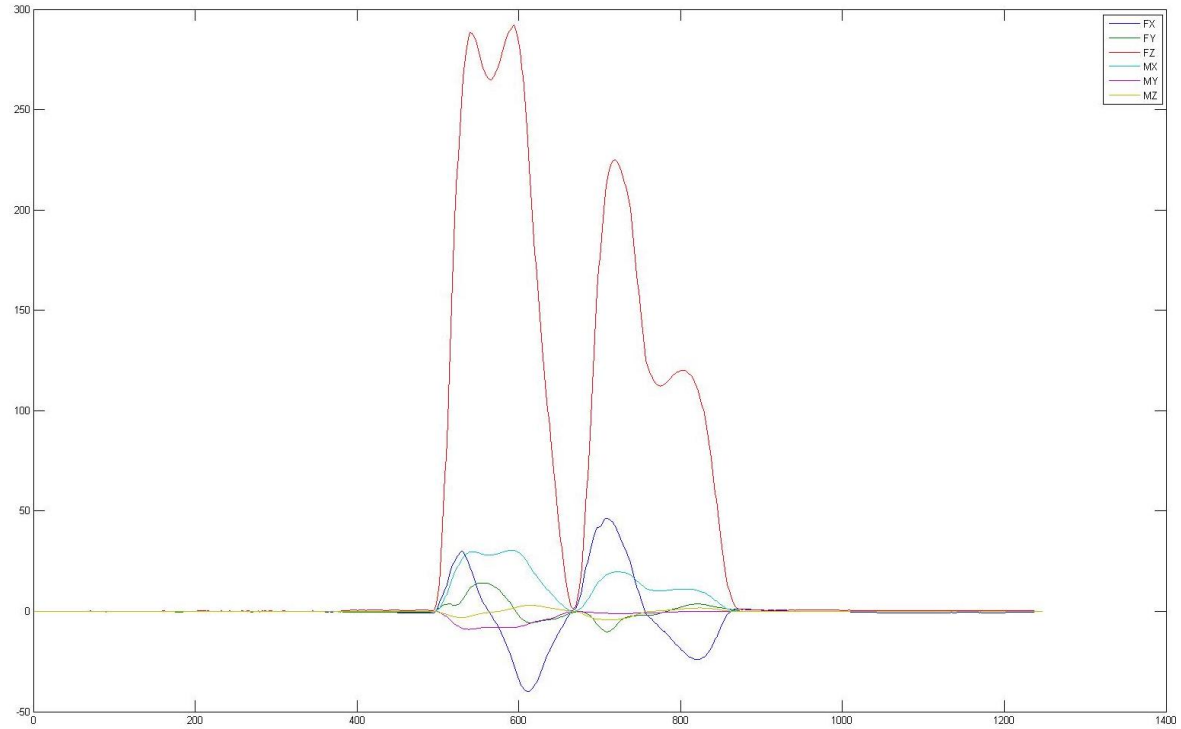

Figure 43. Example of a Filtered Trial

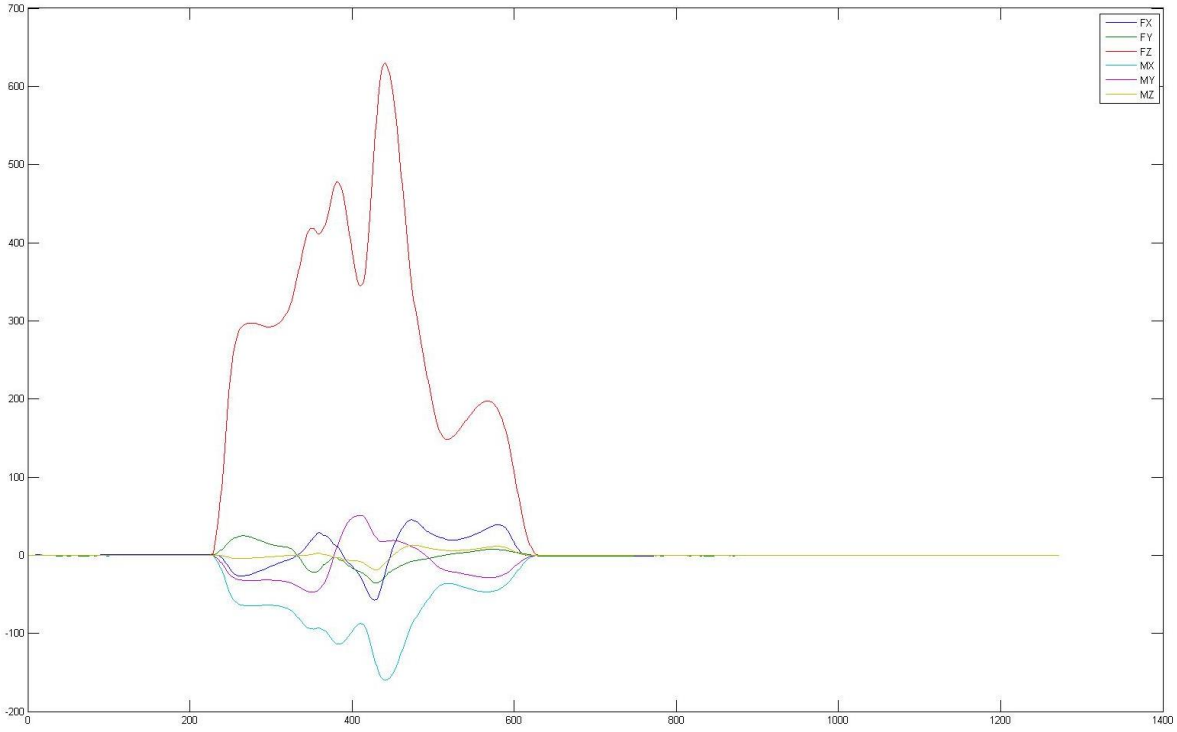

Figure 44. Example of an Excluded Trial – There is a substantial overlap in stance between two or more limbs.

The maximum (max), minimum (min), and range (max – min) were calculated for each trial for all ground reaction forces (FX, FY, FZ) and moment MZ. The differential torque (DT) was calculated by subtracting the absolute value of the min MZ from the absolute value of the max MZ. The magnitude of the push-off force in the X-Y plane was calculated because it is the components that act in the X-Y plane that assist with forward propulsion.[16, 19] The magnitude of the push-off force was calculated by using the equation $a^2 + b^2 = c^2$, where a = average max FX, b = average max FY, and c = magnitude of the push-off force in the X-Y plane. Additionally, the means and standard deviations of each variable were calculated for each day for each pig. Unlike the motion capture system, there was no way to determine velocity using force plate data, so all force plate results at varying velocities are grouped together.

5.3.3 STATISTICAL ANALYSIS

Data presented were analyzed for statistically relevant differences or changes over time where applicable using a Linear Mixed Model (LMM) with a Bonferroni correction. Due to the nature of the data (non-independent, unbalanced, multiple sources of variability, and variability between animals), a LMM was used to adjust for Type I error caused by missing data and to limit the sensitivity of the data to outliers. Statistical significance was defined when $p \leq .05$. LMM was used to test for:

- Differences in FZ between AFL and IFL during PRE
- Differences in FZ between ABL and IBL during PRE
- Changes in weight distribution (FZ) for each leg over time
- Within each velocity category over time, changes in:

- Support and Stance time as %GC for ABL and IBL
- Average stride length for ABL and IBL

- Changes in average max prosthetic angle within each pig over time
- Pearson's correlation between:
  - Velocity and Ankle Angle
  - Velocity and Duration of GC
  - Speed, Ankle Angle, and Duration of GC

5.4 RESULTS – SPECIFIC AIM III

Four Yucatan Mini Pigs completed the study with surgery-to-termination times of 8 weeks (Pig 1), 5 weeks (Pig 2), 11 weeks (Pig 3), and 10 weeks (Pig 4).

5.4.1 DARTFISH RESULTS

5.4.1.1 Trials Collected

Data were collected on Pig #1 at 3, 5, and 8 weeks post-op; Pig #2 at 2 and 5 weeks post-surgery; Pig #3 at 2, 3, 6, 7, 10, and 11 weeks post-surgery; and on Pig #4 at 1, 2, 5, 6, 9, and 10 weeks post-surgery. Figure 45, Table 6, and Table 7 show the distribution of trials collected per quarter.

Figure 45 shows the distribution of data collected from each pig over each quarter. The bottom axis shows the Quarter in which data were collected and the total number of trials for all pigs. Trials collected on Pigs #1, 3, and 4 make up 19.5%, 23.4%, and 21.9% of all PRE trials,

respectively, for a total of 64.8%. Trials collected on Pig #2 make up 35.2% of all PRE trials, which is an average of 64% more than any of the other three pigs.

Table 6 shows the number of trials collected for both the intact and amputated (amp) hind legs at each quarter in each velocity category. Only 4.5% of the trials collected post-op were in velocity category S5 (1.5-1.749 m/s). Q3 contained data from only 50% of the pigs for intact leg and 25% of the pigs for amputated leg. For a more evenly-weighted analysis, velocities above 1.5 m/s (S5, S6, and S7) as well as any data falling in Q3 were not included in analysis with data from all pigs.

Table 7 shows the number of trials collected for both the intact leg and the amputated leg (amp) and the number of experiments (days) over which those trials were collected per animal during each quarter. There were 116 trials collected on Pig #2 pre-op, while none of the other pigs had more than 38 trials collected per leg during PRE. For all weeks in PRE for both hind legs, Pig #1 had total of 64 trials collected, Pig #2 had a total of 116 trials collected, Pig #3 had a total of 77 trials collected, and Pig #4 had a total of 72 trials collected. The average number of trials collected for all animals per experiment in each quarter was 12.7 trials in PRE, 15.8 in Q1, 14.3 in Q2, and 13.9 in Q3. For PRE through Q2, the average number of trials collected per quarter for intact leg was 121.3, and the average for amputated leg was 118.3.

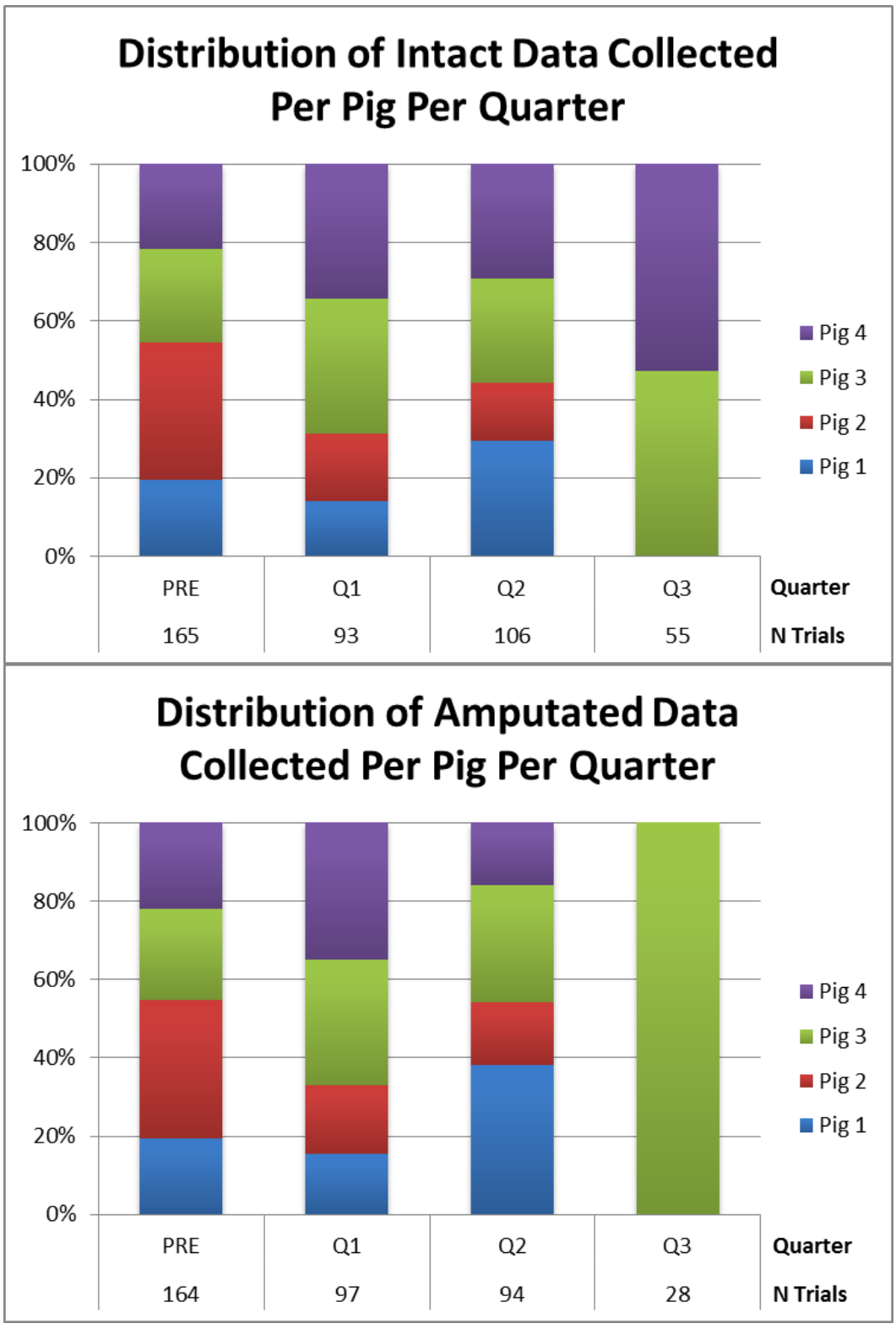

Figure 45. Distribution of all Dartfish trials collected per pig during each quarter for both the intact hind limb (top) and the amputated hind limb (bottom). Q3 is only represented by 1 pig in the "Amputated Data" graph because Pig #4 was not loading prosthetic during weeks 6-10.

Table 6. Number of Trials Collected at Each Velocity Per Quarter for Both Hind Legs: All Pigs Combined

	PRE		Q1		Q2		Q3	
Speed	Intact	Amp	Intact	Amp	Intact	Amp	Intact	Amp
.5-.75 m/s	16	13	13	21	19	15	3	3
.75-1.0 m/s	35	44	39	22	37	29	21	9
1.0-1.25 m/s	28	24	23	31	32	27	29	12
1.25-1.5 m/s	31	35	12	16	13	16	2	4
1.5-1.75 m/s	34	26	6	6	3	6	0	0

Table 7. Distribution of Collection Days and Number of Trials Collected: Broken Down by Each Pig for Both the Intact Hind Limb and the Amputated Hind Limb Over Each Quarter

	PRE			Q1			Q2			Q3		
Pig	Days	Intact	Amp	Days	Intact	Amp	Days	Intact	Amp	Days	Intact	Amp
#1	3	33	32	1	13	15	2	31	36	X	X	X
#2	4	58	58	1	16	17	1	16	15	X	X	X
#3	3	38	38	2	32	31	2	28	28	2	26	28
#4	3	36	36	2	32	34	2	31	15	2	29	0

5.4.1.2 Stride Length

Figure 46 shows the average stride lengths of all pigs over time at each speed. For both legs at all velocities except S4, stride lengths at Q2 were less than those at PRE. The stride lengths in meters for S1-S4 of the intact leg during PRE were 0.574, 0.606, 0.649, and 0.676, respectively. The stride lengths in meters for S1-S4 of the intact leg during Q2 were 0.565, 0.591, 0.626, and 0.688, respectively. The change in stride length from PRE to Q2 at S1 decreased by 1.5% for the IBL and decreased by 3.5% for the ABL. The greatest difference in stride length from PRE to Q2 was found at S3 for IBL and at S2 for ABL, with a decrease of 3.6% and 3.9%, respectively. Variation of stride length of the ABL was the greatest in Q2 for all

speeds. Change in standard deviation of the ABL for S1-S4 increased from PRE to Q2 by 82.1%, 100.2%, 83.6%, and 54.0%, respectively. For the IBL during Q2, there was less variance than during PRE in stride length at S3 and S4, with a decrease of only 9.66% and 8.78%, respectively.

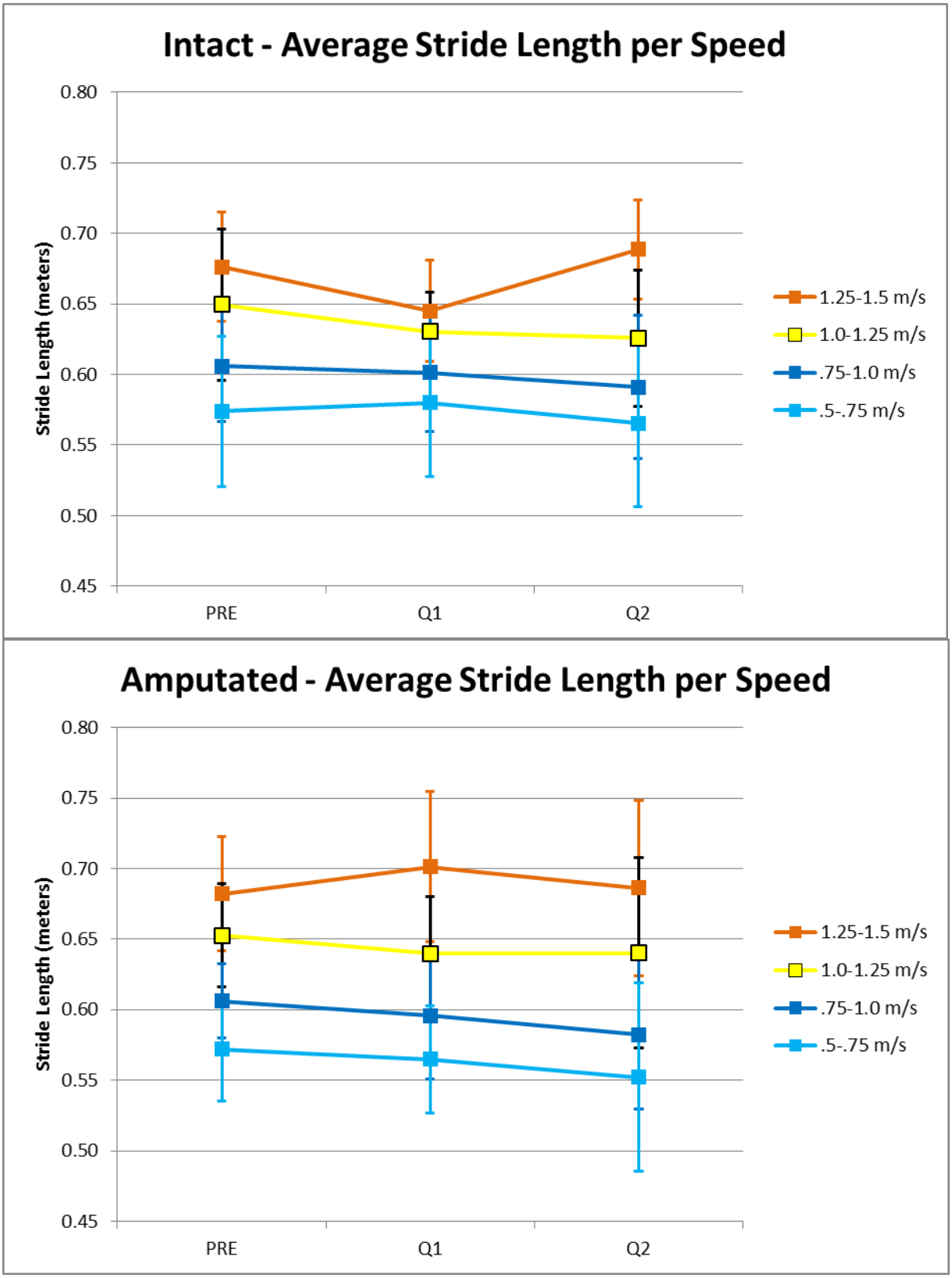

Figure 46. Average stride length per velocity for intact and amputated hind legs. error bars represent one standard deviation.

5.4.1.3 Gait Cycle

GC is the time difference from touchdown to touchdown or from liftoff to liftoff for each of the back limbs. Figure 47 displays the average duration of GC during each quarter for each speed. The slowest velocity (S1) has consistently the longest duration of GC over each quarter for both hind limbs. Oppositely, the shortest duration of GC was found at S4 for both hind limbs. The variance in duration GC was greatest for the intact limb at S1 for all quarters, with a maximum standard deviation of 13% of the average duration GC occurring during Q2. The average deviation from the mean increased over time at all velocities for the ABL.

Figure 48 shows the relationship between stride length, duration of GC, and velocity for quarters PRE, Q1, and Q2. For the IBL, the average duration of GC in seconds for S1-S5 was 0.856, 0.693, 0.573, 0.496, and 0.452, and the corresponding average stride length in meters for each of those velocities was 0.573, 0.599, 0.635, 0.670, and 0.728, respectively. The average duration of GC of the IBL decreased by 0.163 seconds from S1 to S2, 0.120 seconds from S2 to S3, 0.077 seconds from S3 to S4, and 0.044 seconds from S4 to S5. The average stride length in meters of the IBL increased by 0.026 from S1 to S2, 0.036 from S2 to S3, 0.035 from S3 to S4, and 0.059 from S4 to S5. Similarly, the average duration of GC across each velocity group for the ABL decreased by 0.167, 0.096, 0.083, and 0.055 seconds, respectively; the average stride length of the ABL increased by 0.032, 0.049, 0.046, and 0.029 meters, respectively.

GC duration is the sum of support time (touchdown to liftoff) and swing time (liftoff to touchdown). Representing each speed, Figure 49, Figure 50, Figure 51, and Figure 52 show how

support time and swing time as an average across all pigs changed in relationship to one another between intact and amputated hind legs. There was no significant change between support and swing time over the study for the intact leg at velocity S1, but there was at least one quarter in all other velocities where support vs. swing of the intact leg was significantly different than pre-op. At velocities S1 and S2, the amputated stance time was significantly less in all quarters post-op.

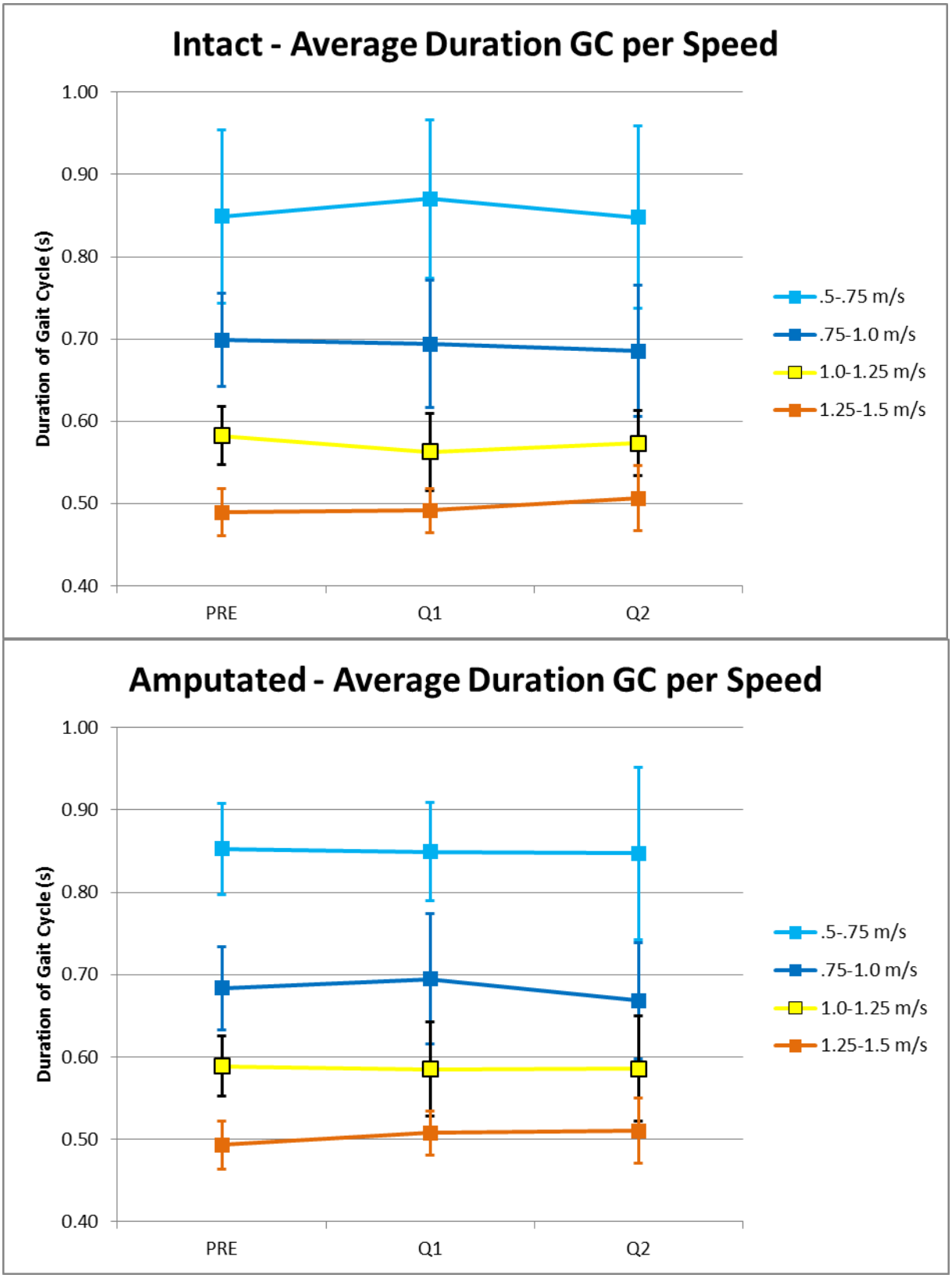

Figure 47. Average duration of gait cycle separated by velocity over each quarter. Error bars represent one standard deviation.

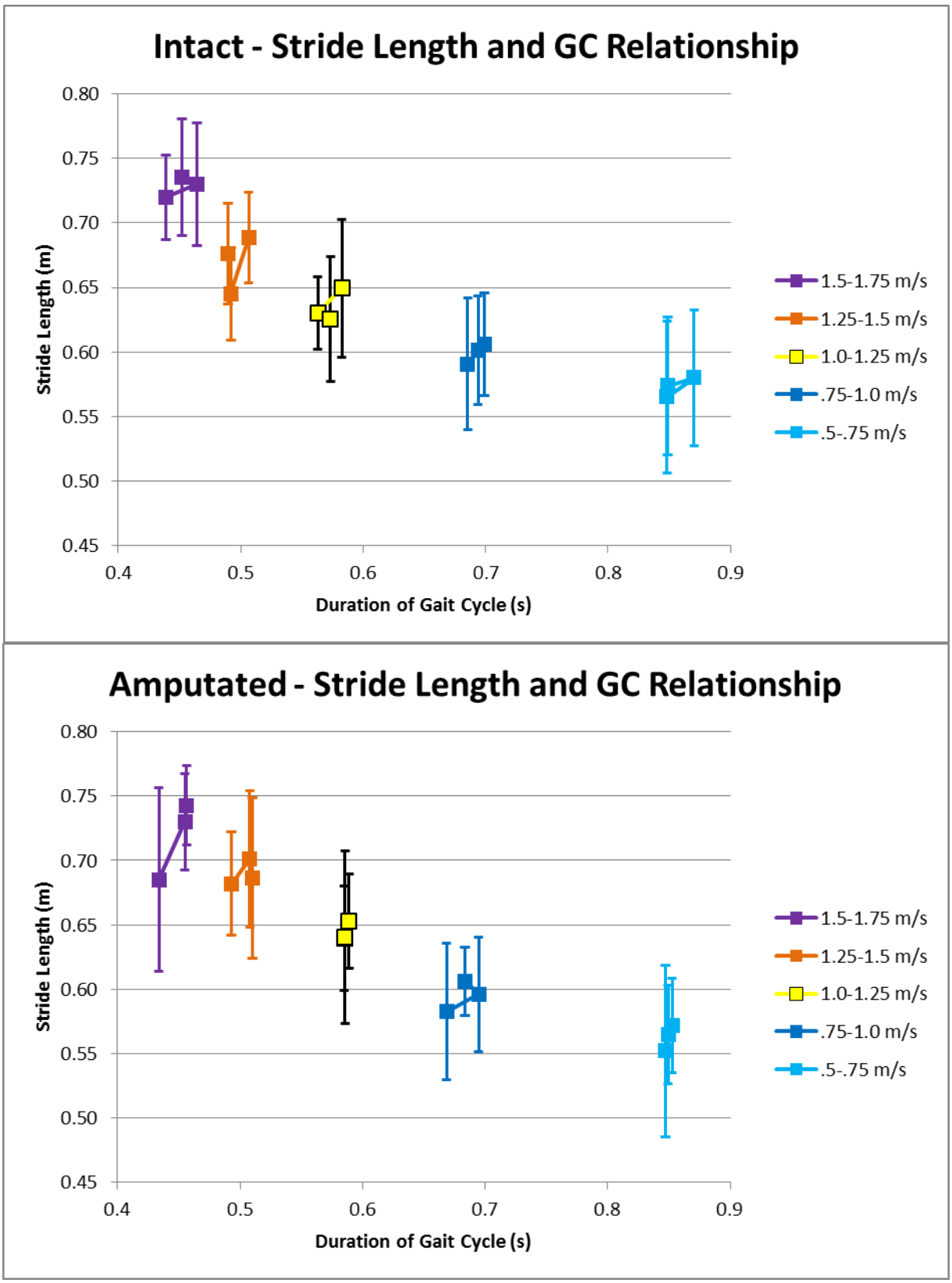

Figure 48. Strong correlation between speed, stride length, and duration of gait cycle. Quarters PRE, Q1, and Q2 are represented within each speed. Error bars represent one standard deviation.

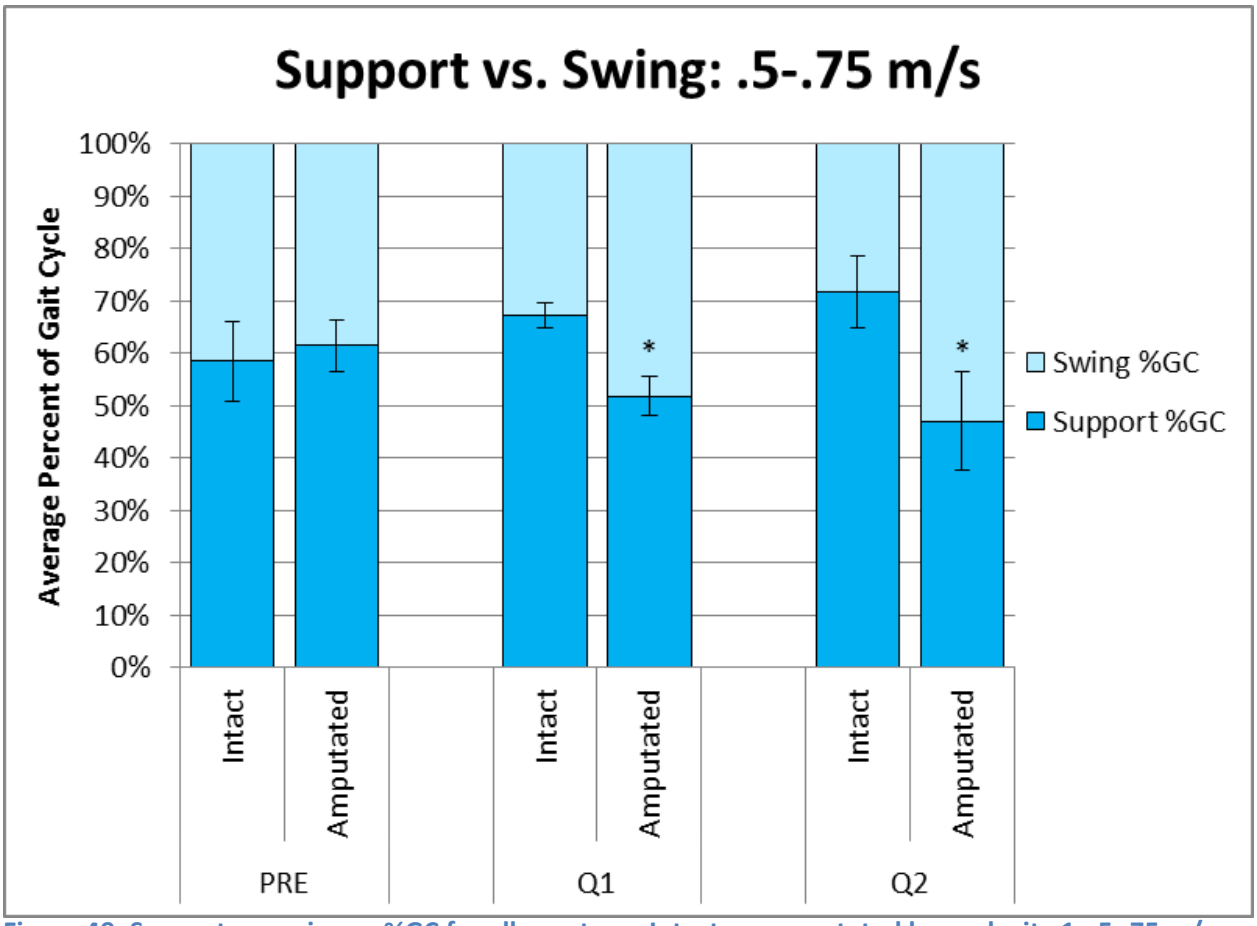

Figure 49. Support vs. swing as %GC for all quarters - Intact vs. amputated leg; velocity 1: .5-.75 m/s
***Denotes significant change compared with PRE (p<.05)**
Error bars represent one standard deviation.

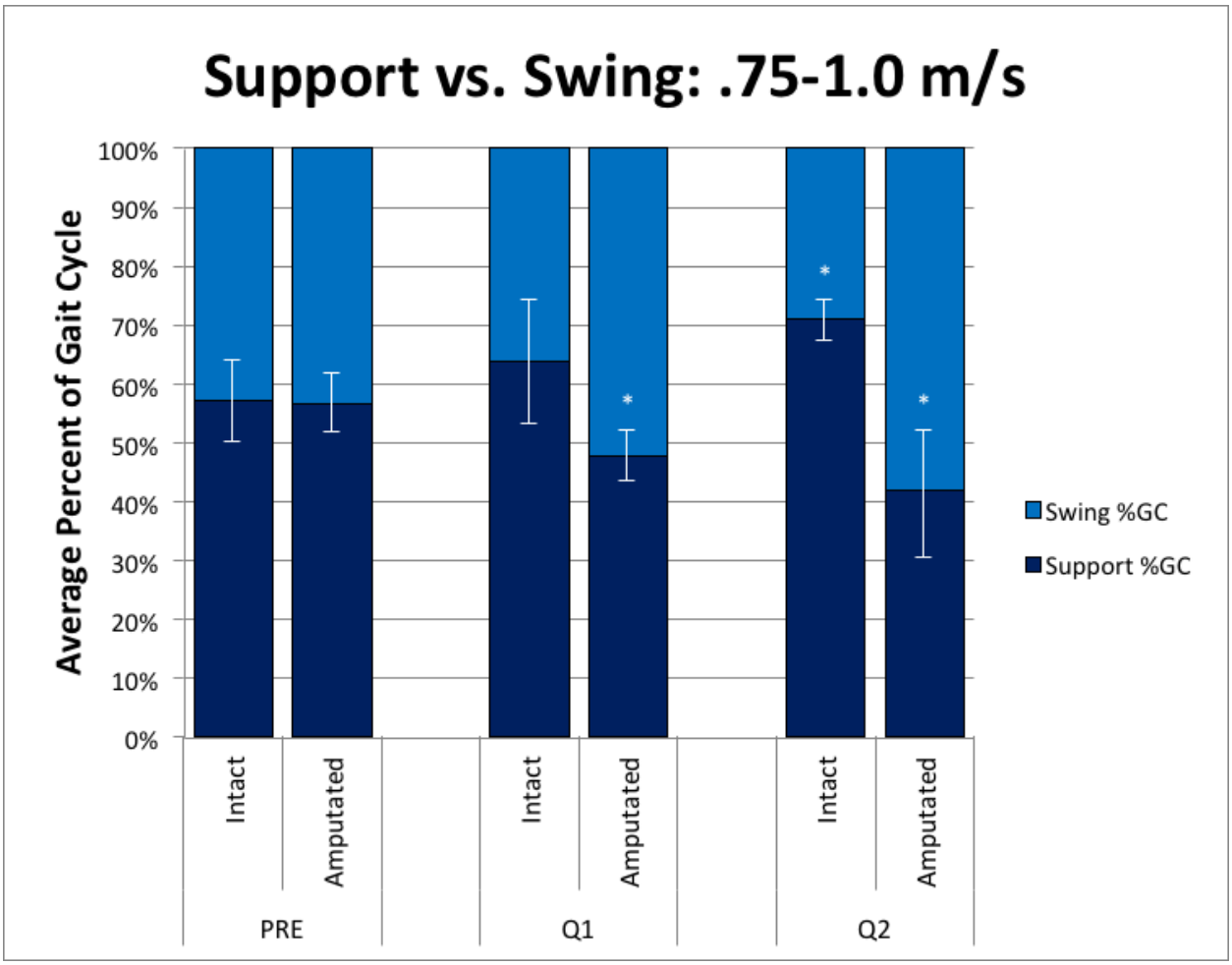

Figure 50. Support vs. swing as %GC for all quarters - Intact vs. amputated leg; velocity 2: 0.75-1.0 m/s
***Denotes significant change compared with PRE (p<.05)**
Error bars represent one standard deviation.

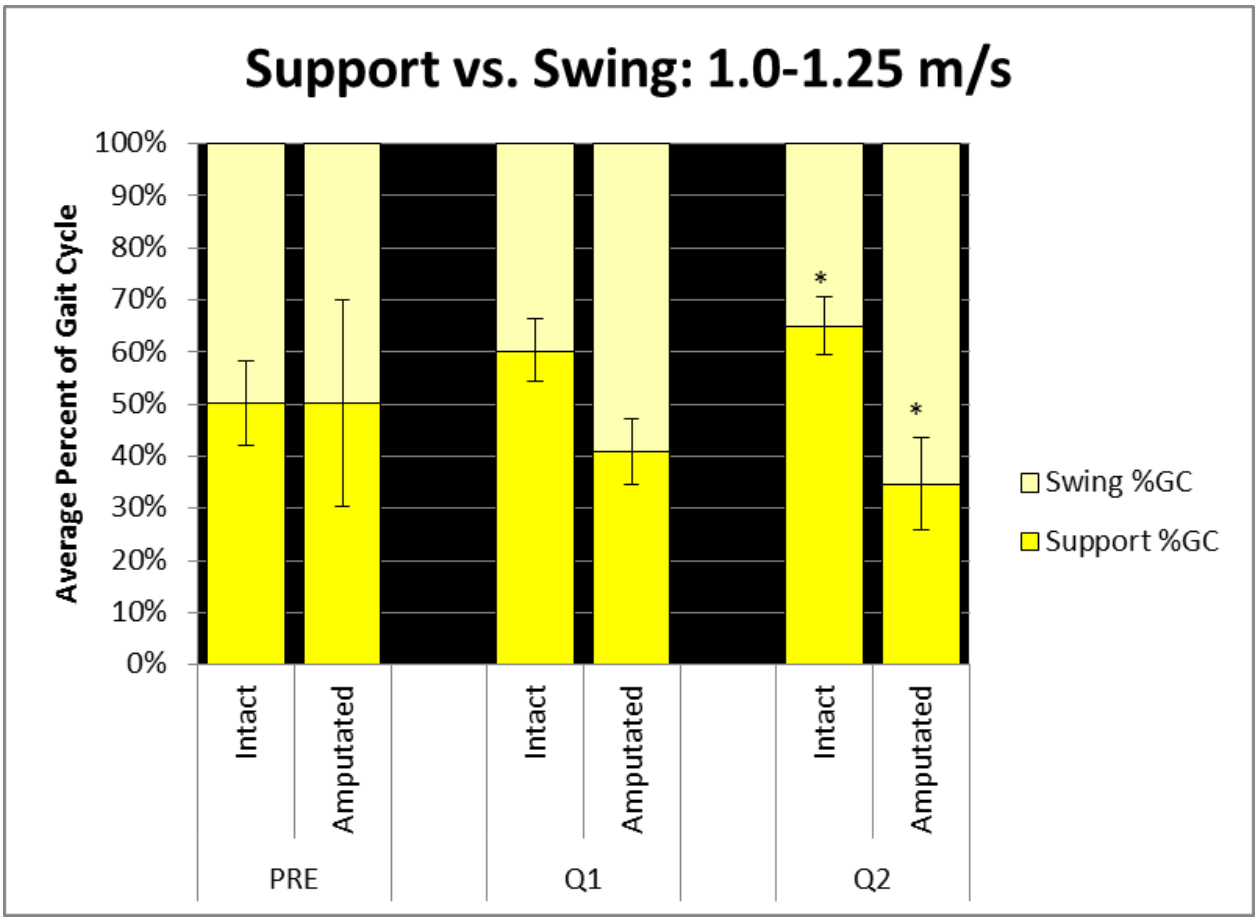

Figure 51. Support vs. swing as %GC for all quarters – intact vs. amputated leg; velocity 3: 1.0-1.25 m/s
***Denotes significant change compared with PRE (p<.05)**
Error bars represent one standard deviation.

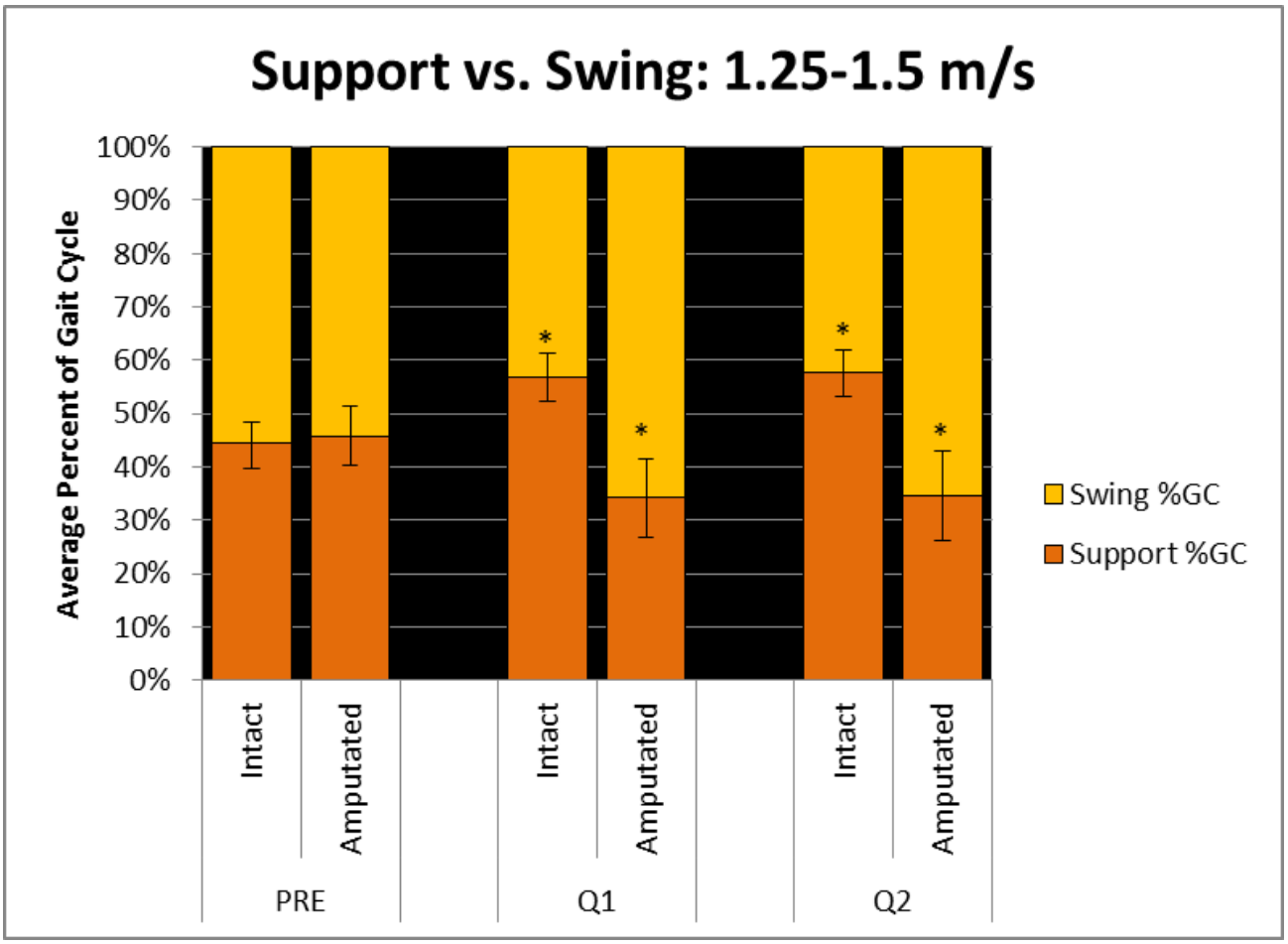

Figure 52. Support vs. swing as %GC for all quarters - intact vs. amputated leg; velocity 4: 1.25-1.5 m/s
***Denotes significant change compared with PRE (p<.05)**
Error bars represent one standard deviation.

5.4.1.4 Speed

Trial distribution by velocity category as a percent of all trials collected are shown per pig in Figure 53 and as an average of all pigs in Figure 54. Of the 32 trials collected from each hind leg during PRE for Pig #1, S5 contained 34.37% of the trials for the intact limb and 31.25% of the trials for the amputated limb. The frequency of trials in velocity category S5 during Q1 for the intact and amputated hind limbs dropped to 15.38% and 7.14%, respectively. For Pig

#2, trials collected during PRE were most frequently within S5 for IBL and S4 for ABL, making up 37.77% and 27.59% of trials collected, respectively.

Velocity category S4 contained 25% of trials collected during both Q1 and Q2 for the ABL, but contained 29% and 20% for Q1 and Q2, respectively, for the IBL. Pig #3 evenly distributed trials collected during PRE within S2 and S3, making up 68.42% of all trials collected pre-op for the ABL. S3 for Pig #3 contained 38.46%, 25.00%, 28.57%, and 84.62% of the trials collected in PRE, Q1, Q2, and Q3, respectively for the IBL. Pig #4 prefered velocities within S1 or S2 on both hind legs during the entire study, with 71.76% of all IBL trials and 59.08% of all ABL trials falling within those velocity categories for a total of 65.42% of all trials collected on Pig #4.

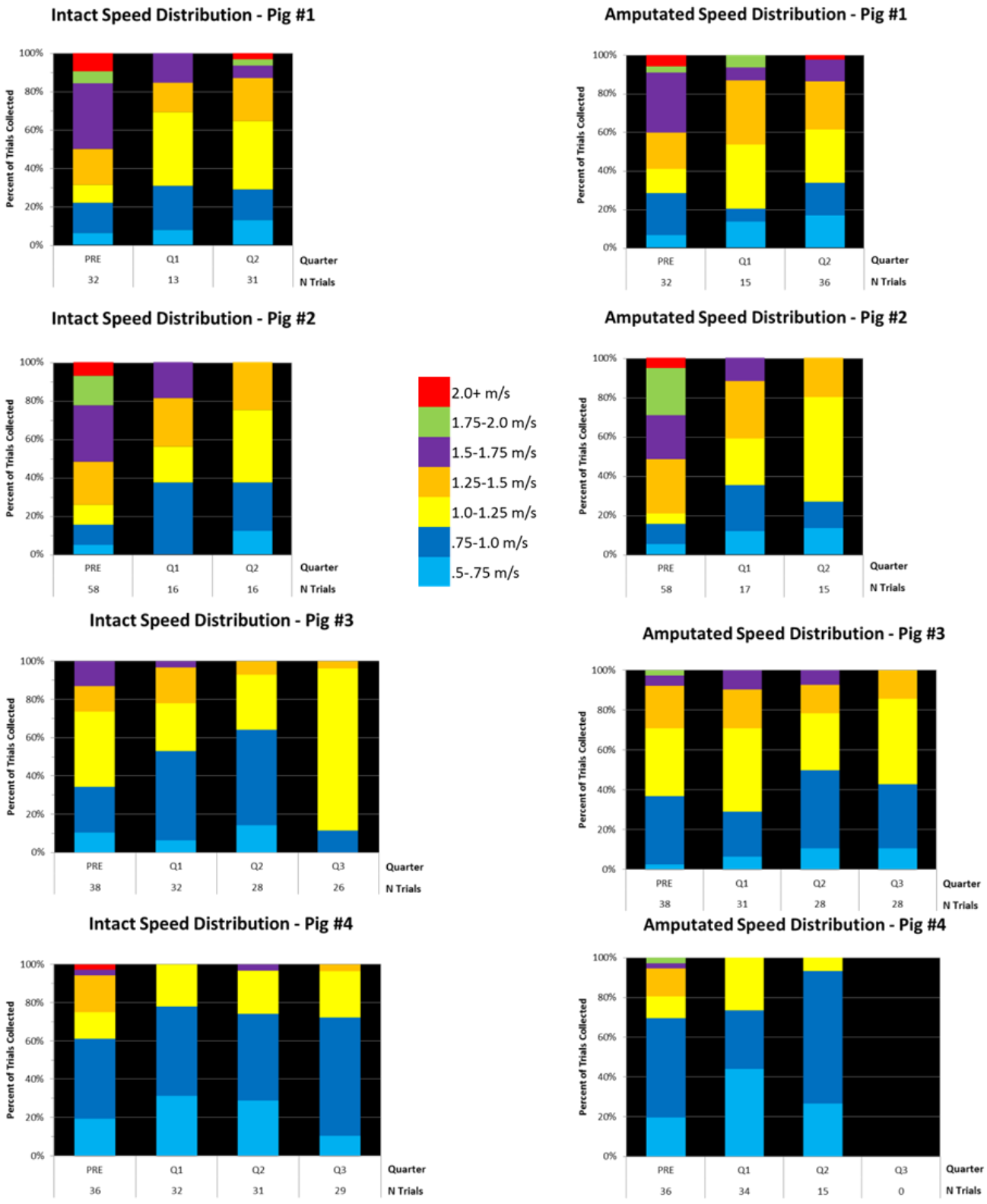

Figure 53. Velocity distribution collected for the intact hind limb (left) and amputated hind limb (right) per pig per quarter.

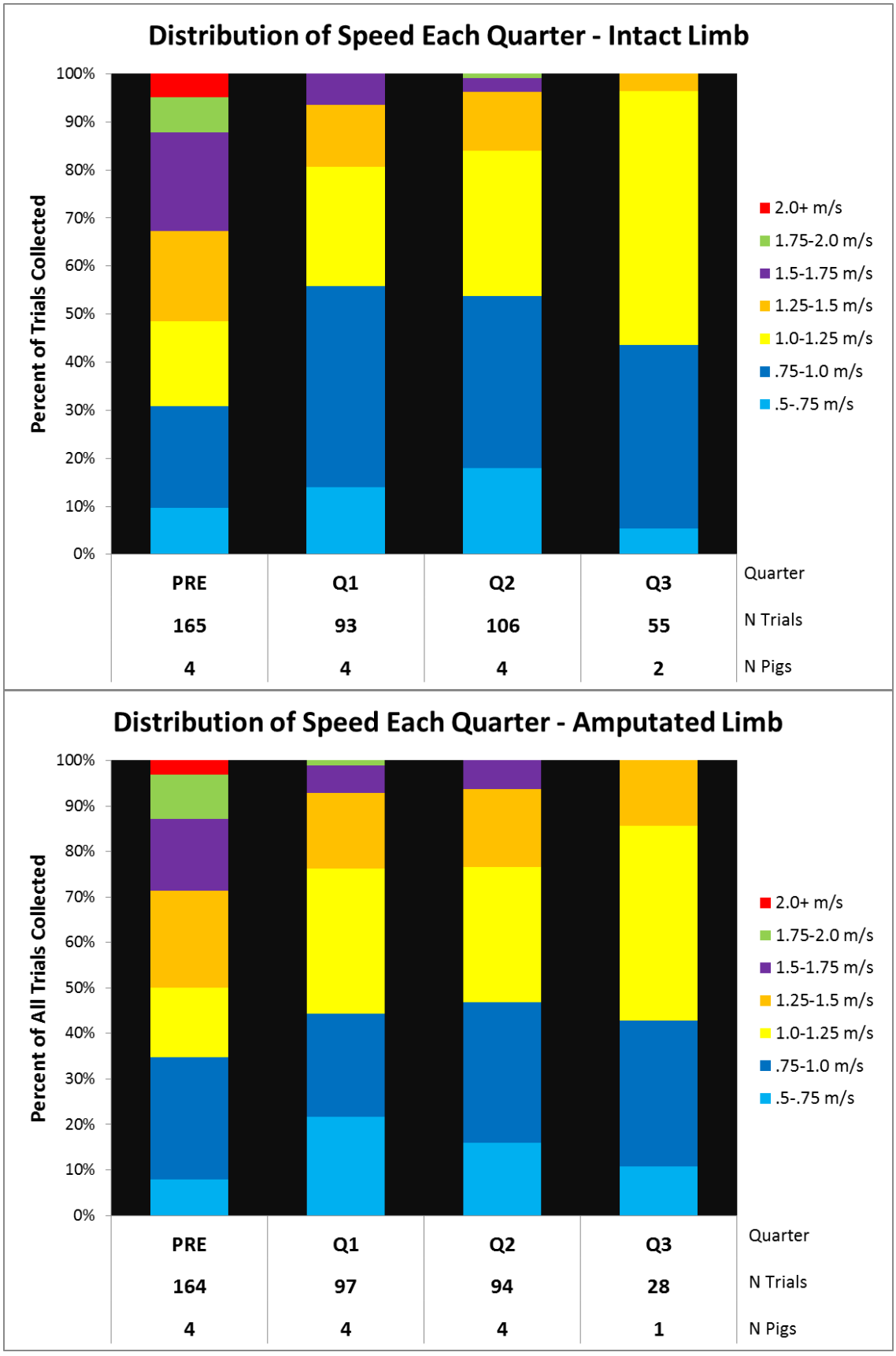

Figure 54. Distribution of trials collected per velocity for each quarter for both the intact hind limb (top) and the amputated hind limb (bottom).

Table 8 and Table 9 show the Pearson Correlation Coefficients (R) between speed, stride length, and duration of GC for the intact leg and the amputated leg, respectively. R was negative when relating duration of GC to both velocity and stride length for both legs: for IBL, the R values were -0.85635 and -0.36939, respectively; for ABL, the R values were -0.88565 and -0.47154, respectively. Stride length and velocity had a positive R for both IBL and ABL of 0.69059 and 0.728682, respectively. For all correlations, p≤.0001.

Table 8. Pearson Correlation between Speed, Stride Length, and Duration of GC for INTACT Leg

Pearson Correlation Coefficients, N = 396 Prob < |r| under H0: Rho = 0			
	Speed	**Stride Length**	**Duration of GC**
Speed	1.00000	0.69059 <.0001	-0.85635 <.0001
Stride Length	0.69059 <.0001	1.00000	-0.36939 <.0001
Duration of GC	-0.85635 <.0001	-0.36939 <.0001	1.00000

Table 9. Pearson Correlation between Speed, Stride Length, and Duration of GC for AMPUTATED Leg

Pearson Correlation Coefficients, N = 359 Prob < |r| under H0: Rho = 0			
	Speed	Stride Length	Duration of GC
Speed	1.00000	0.72682 <.0001	-0.88565 <.0001
Stride Length	0.72682 <.0001	1.00000	-0.47154 <.0001
Duration of GC	-0.88565 <.0001	-0.47154 <.0001	1.00000

5.4.1.5 Prosthetic Angle

As discussed in 5.3.1.4 Prosthetic Angle, prosthetic angle was measured by the angle the prosthesis made with the ground as shown in Figure 40. Max prosthetic angle was defined as the prosthetic angle at touchdown, and the min prosthetic angle was defined as the prosthetic angle right before liftoff. The prosthetic angle range was calculated by finding the difference between the max and min prosthetic angle.

Figure 55 shows a visual representation of how the max ankle angle changed over time with each pig at each speed. To show a more accurate visual representation of the change over time, the angle is reported as an average per velocity per pig for every experiment post-surgery. Each color represents a different pig, and each line within that color is a different speed.

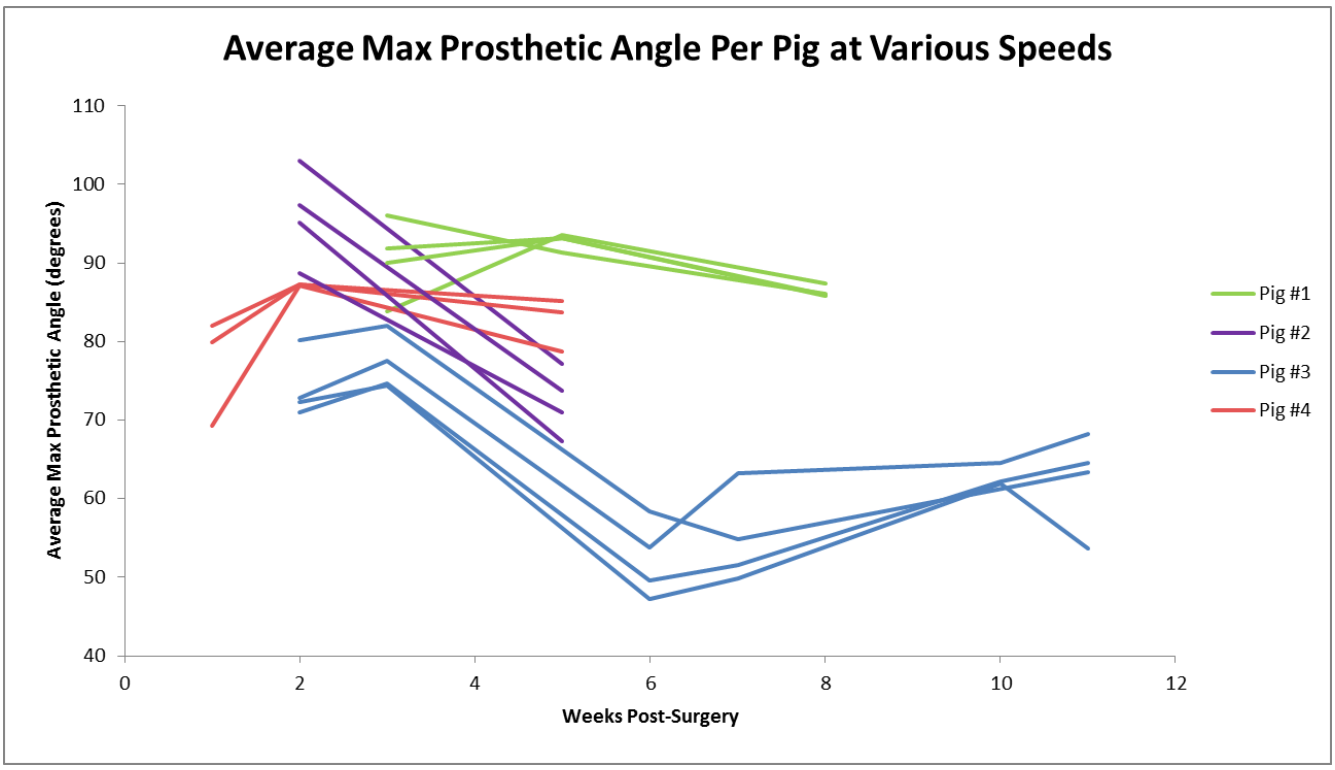

Figure 55. Average max prosthetic angle per pig at various velocities .5-1.5 m/s in .25 m/s increments.

The average max prosthetic angle for Pig #1 at Q1 was 90.4° and at Q2 was 89.7°. For Pig #2, this angle was 96.1° and 72.3° for Q1 and Q2, respectively. Pig #3 had an average maximum prosthetic angle of 75.6°, 53.6°, and 62.7° for Q1, Q2, and Q3, respectively, while Pig #4's maximum prosthetic angle was 61.6° for Q1 and 61.9° for Q2. Pig #4 stopped loading prosthetic during Q2, so no data were collected on his ABL during Q3. The prosthetic angle ranges of all animals during [Q1 and Q2] at S1-S4 were [42.6°, 38.7°], [40.4°, 34.7°], [36.6°, 32.3°], and [31.7°, 31.7°], respectively.

5.4.1.6 Example Gait

Using estimated markers in approximate anatomical positions shown in Figure 41 and Figure 42, pre- and post-surgery gait examples from Pig #4 are shown in Figure 56 and Figure

57, respectively. To show how they differ from each other, the post-surgery data is superimposed on the pre-surgery data in Figure 58. There were only 4 marker locations used post-surgery while there were 6 marker locations used pre-surgery. The stride length from the GC shown in Figure 56 was .45 meters. A total of 1.25 seconds is represented in Figure 56, while the same number of GCs is represented by only 0.85 seconds in Figure 57. The average vertical movement in mm of each marker (A-F) pre-op was 35.4, 50.7, 73.3, 59.9, 42.1, and 49.5, respectively. The average vertical movement in mm of each marker (A-D) post-op was 39.1, 66.0, 37.9, and 45.8, respectively.

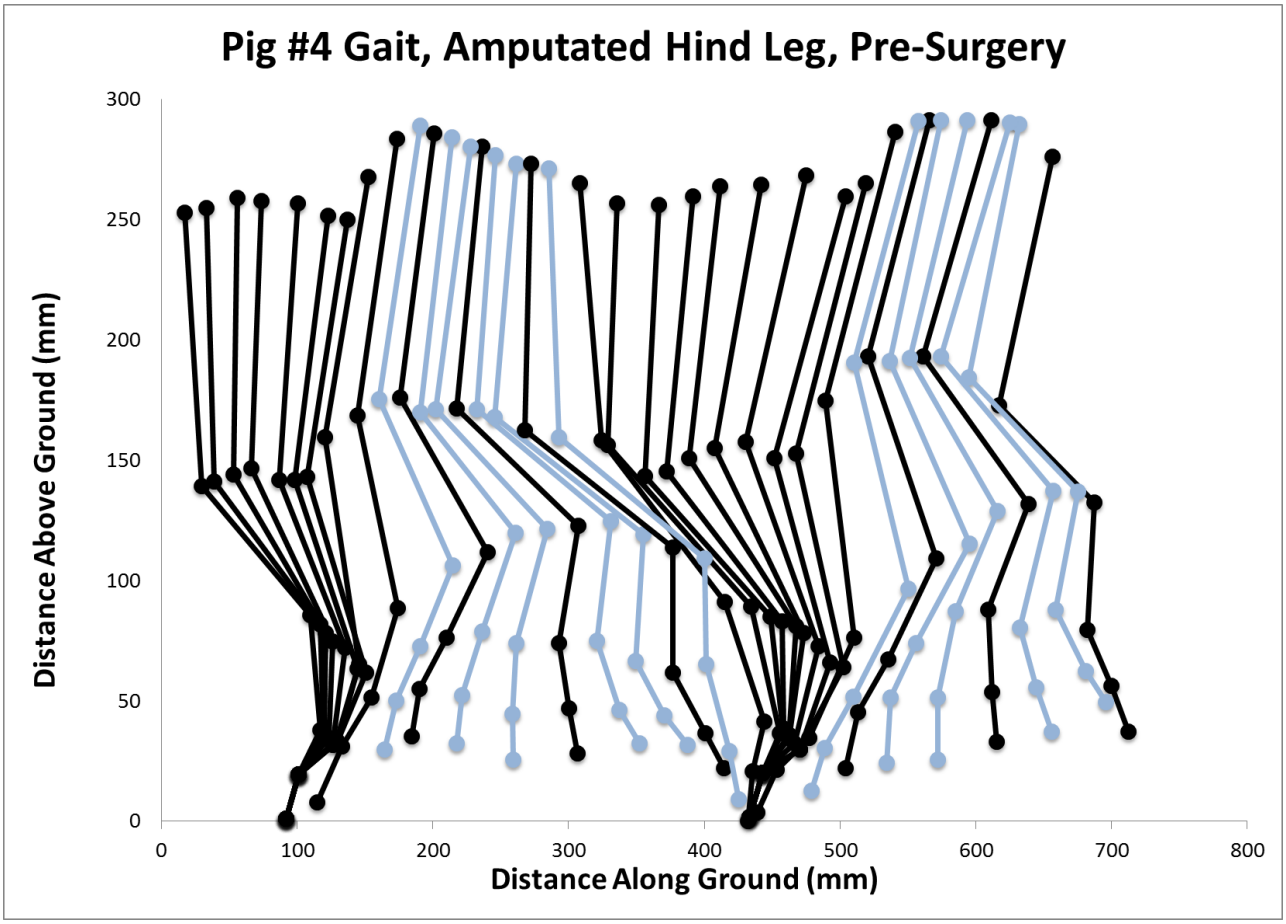

Figure 56. Visual example of gait pre-surgery. All black segments are .05s intervals, while those shown in blue are various intervals between .05s to show trend more easily.

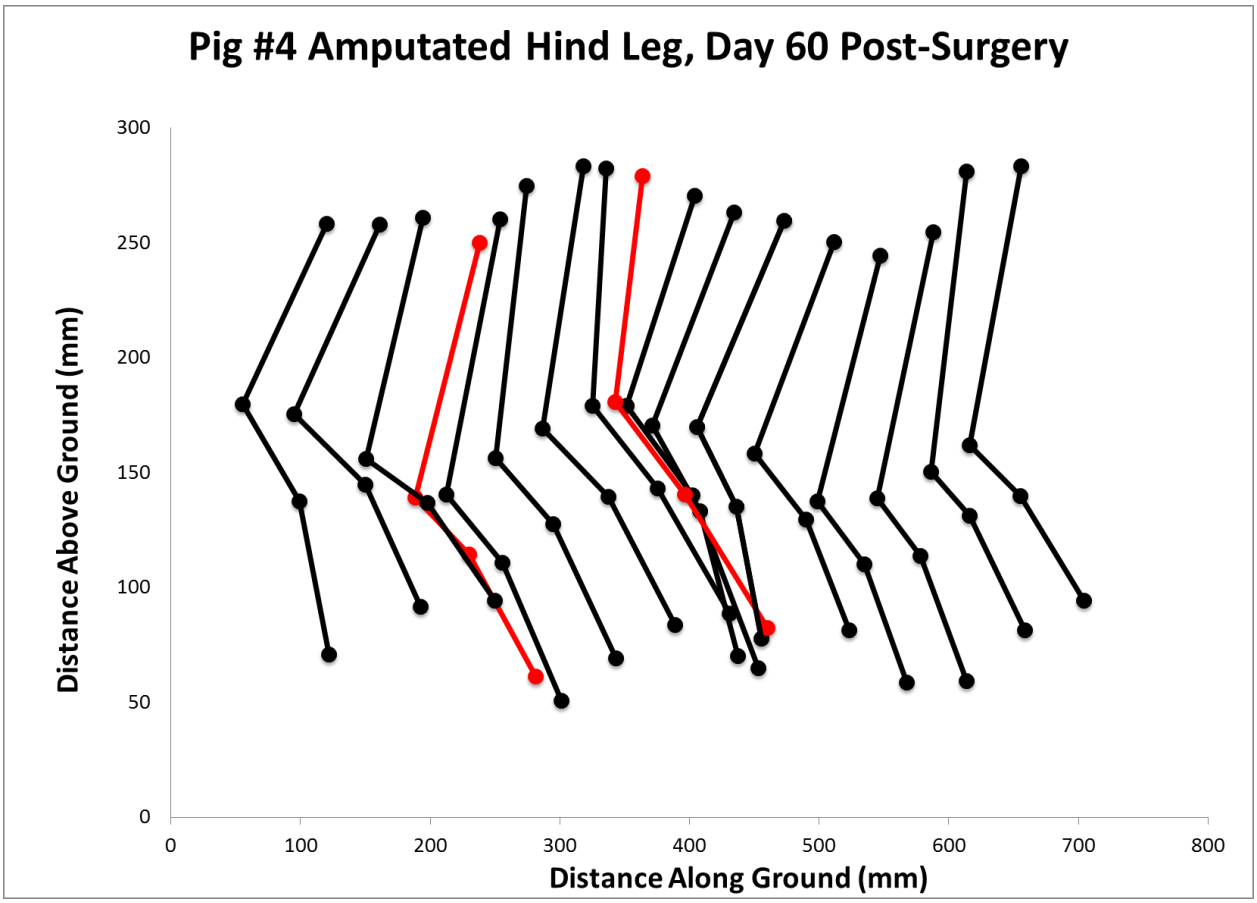

Figure 57. Visual example of gait post-amputation at .05s intervals. Data shown in red overlaps other data and is only a different color to make the trials more easily distinguishable. Pig #4 did not touch down with prosthetic on day 60 post-surgery.

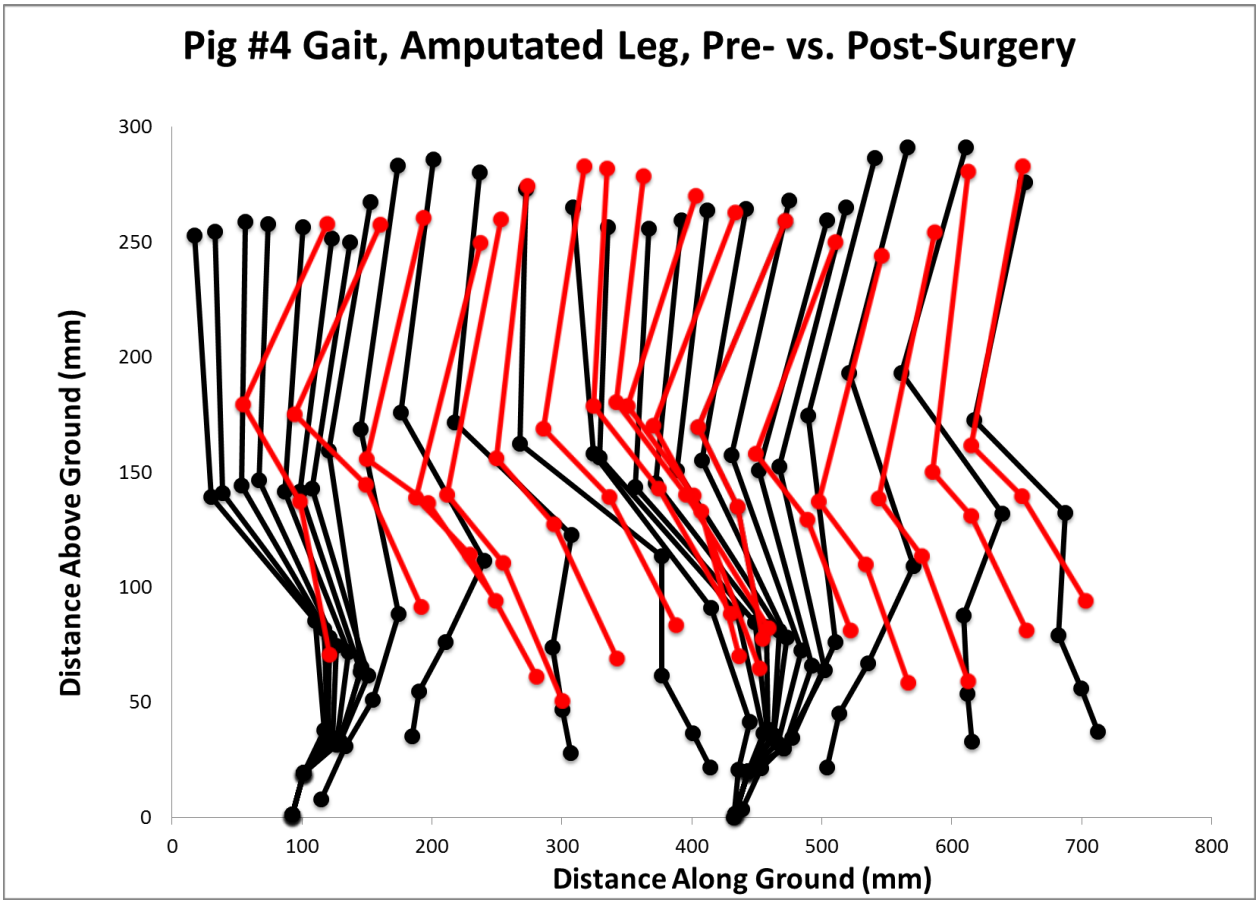

Figure 58. Comparison of Pre- (BLACK) and 60-day post-surgery (RED) visual gait information for Pig #4. All data shown is at .05s intervals.

5.4.2 FORCE PLATE RESULTS

5.4.2.1 Torque

Figure 59 shows the average max (max) and average min (min) torque (MZ) for only Pig #1. The max and min MZ measured in Newton*meters (Nm) during PRE for the ABL was 3.35 and -3.08, respectively. For the IBL during PRE, the max and min MZ were 1.87 and -2.73, respectively. Post-op, ABL had a max MZ of 2.39, 2.43, and 2.62 for weeks 3, 5, and 8, respectively, and min MZ values of -0.59, -1.30, and -1.05. Post-op MZ values for IBL were 0.62,

0.64, and 2.99 for max, and -5.04, -5.77, and -4.07 for min during weeks 3, 5, and 8, respectively.

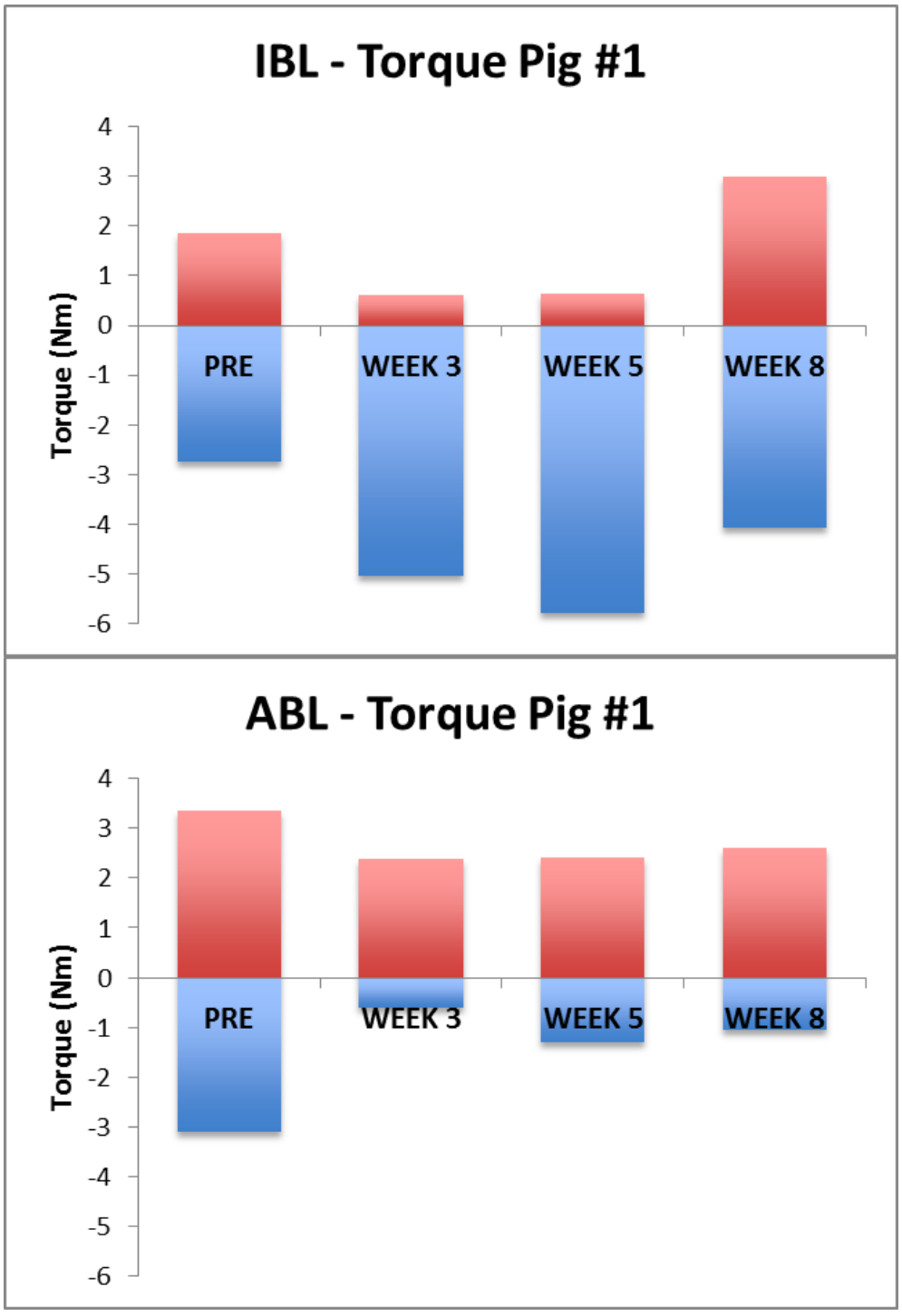

Figure 59. MZ torque, Pig #1. This is the average max (red) and average min (blue) torque found over the course of the study from only Pig #1, whose left leg was amputated. Both the IBL (top) and the ABL (bottom) are shown for each quarter.

Positive torque (red) = unscrewing of the implant
Negative torque (blue) = screwing in of the implant

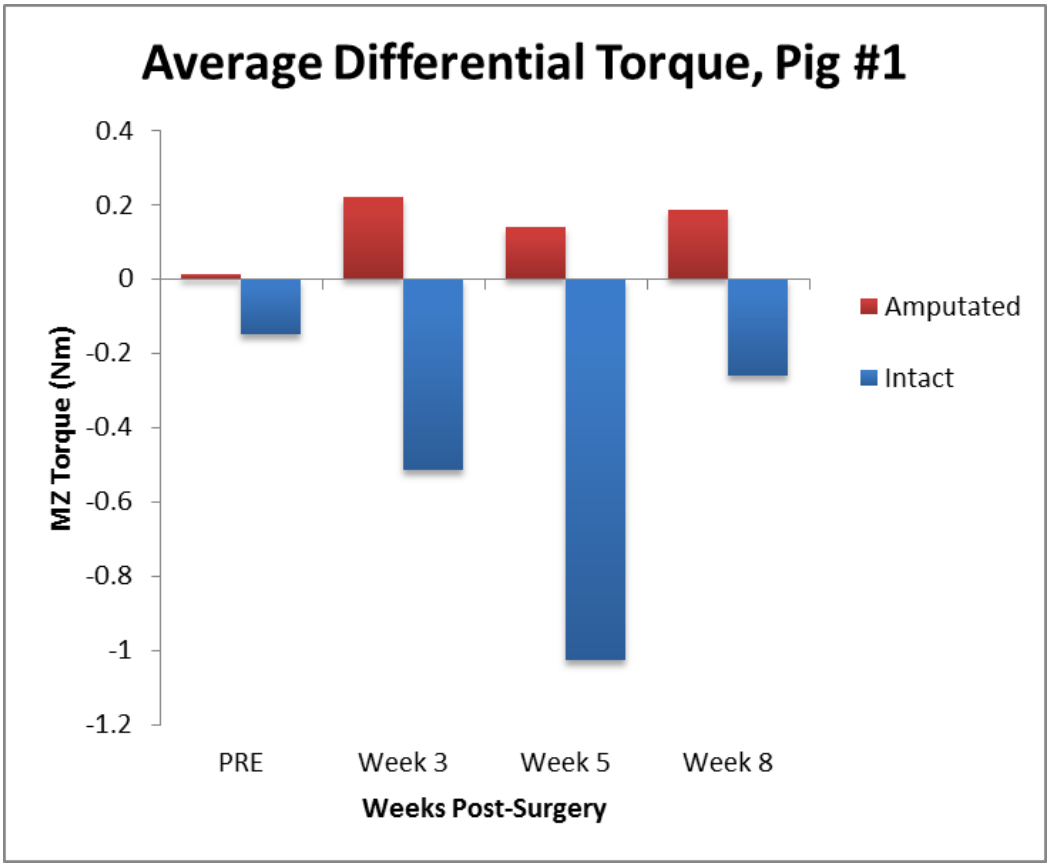

Figure 60. Differential average MZ torque, Pig #1. This is the average torque found over the course of the study from only Pig #1, whose left leg was amputated.

Positive torque (red) = unscrewing of the implant, amputated leg
Negative torque (blue) = represents screwing in of the implant, had the implant been implanted into the intact leg

The differential MZ torque (DT) was calculated by taking the absolute values of the average max and average min and subtracting the min value from the max. The average DT values are shown in Figure 60 for both the ABL (red) and the IBL (blue) for Pig #1. DT during PRE for IBL was -0.151 and for ABL was 0.0134. DT for IBL at weeks 3, 5, and 8 was -0.515, -1.03, and -0.262, respectively. DT for ABL at weeks 3, 5, and 8 was 0.221, 0.140, and 0.185, respectively.

5.4.2.2 Vertical Ground Reaction Force

Vertical ground reaction force (GRF), FZ, was collected and analyzed for each leg. It was first normalized to %BW and then compared across the duration of the study for each animal [Figure 61]. The average difference in GRF of the IFL and AFL as %BW of each pig was 5.5, 4.2, 2.4, and 5.9, respectively, for an overall average difference of 4.49%BW. The average difference in GRF of the IBL and ABL as %BW of each pig was 3.1, 1.4, 0.43, and 0.91, respectively. The GRF of the amputated back leg decreased significantly from PRE in each pig as shown in Table 10. Additionally, the majority of the intact side front leg weight distribution increased significantly from PRE, a combined average of 18.28%. Figure 62 is a combined 100% stacked column representation of the average weight distribution of all trials for each quarter. Pre-surgery, the front legs bore approximately 60% of the weight evenly between the two, and the back two legs evenly bore the remaining 40% during ambulation.

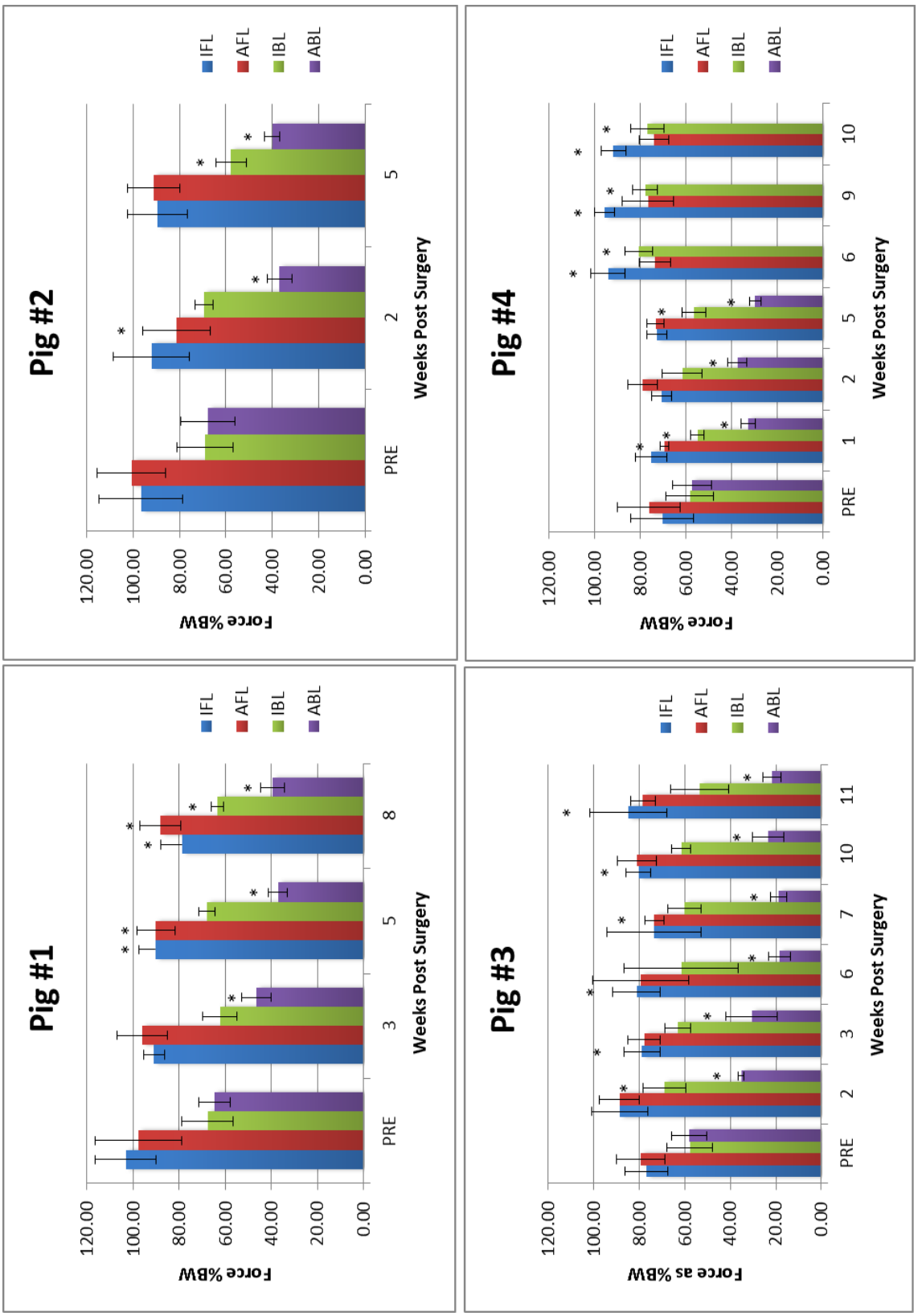

Figure 61. GRF averages of all trials for each leg for each pig over time.
***Denotes significant change between pre- and post-surgery average GRF values indicated ($p<.05$). Error bars represent one standard deviation.**

Table 10. Percent Change in Percent Weight Distribution from Pre-Surgery to Last Collection Post-Surgery.

	FRONT INTACT	FRONT AMPUTATED	BACK INTACT	BACK AMPUTATED
PIG #1	13.62	8.62	-1.16	-30.1
PIG #2	-5.42	10.41	15.76	-23.98
PIG #3	25.85	12.48	4.73	-56.75
PIG #4	39.07	6.59	39.06	-100

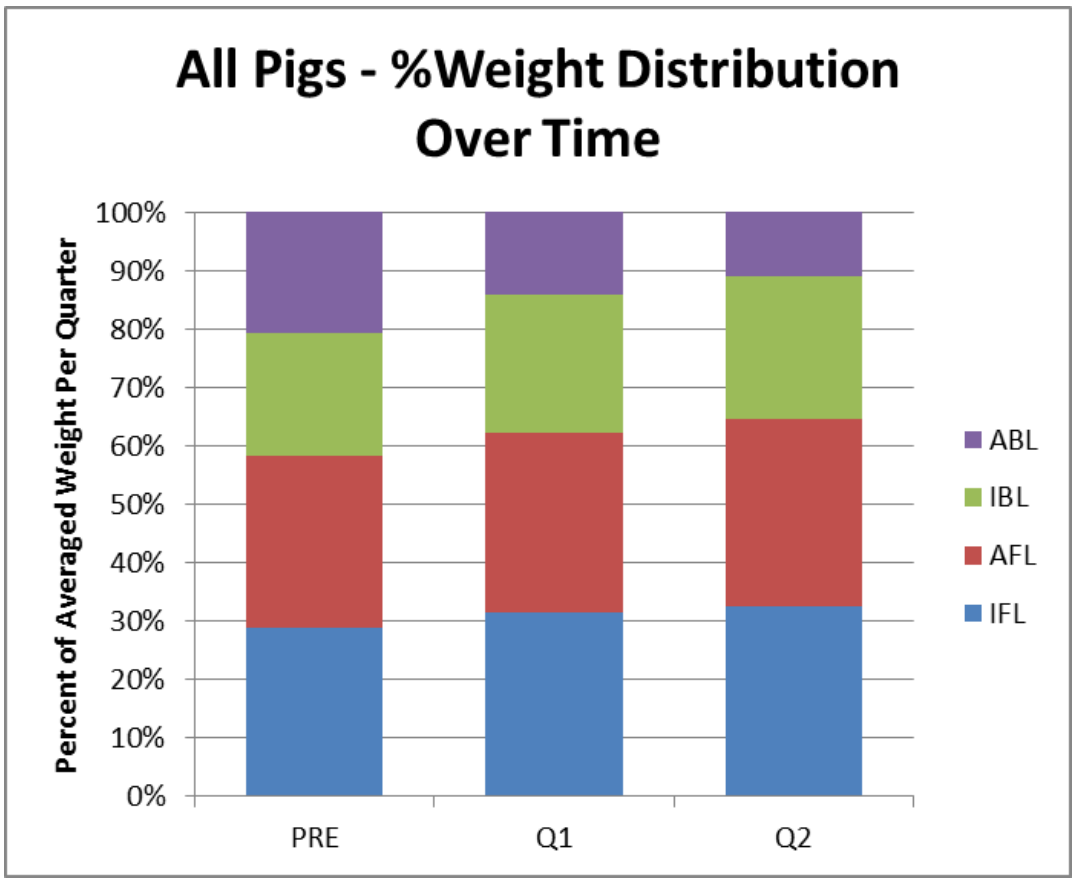

Figure 62. GRF per leg - distribution across all legs for quarters PRE, Q1, and Q2.

Broken down by pig in Table 11, Pigs #2-4 had greater %BW distribution to their AFL than their ABL, while Pigs #1, 2, and 4 distributed more BW on the IBL than their ABL. The

average max GRF as %BW during Q2 for Pigs #1 and 2 was greater for their AFL than their IFL, while Pigs #3 and 4 distributed more BW to their IFL than their AFL.

The combined average max GRF for all AFL and IFL of all pigs across PRE, Q1, and Q2 were [88.52, 86.77], [83.67, 84.94], and [82.58, 83.69] %BW, respectively. The combined average max GRF for all ABL and IBL of all pigs across PRE, Q1, and Q2 were [61.97, 63.21], [37.87, 63.99], and [27.95, 63.24] %BW, respectively. The GRF for average ABL decreased by almost 39% from PRE to Q1, and by 26% from Q1 to Q2. The total decrease in average max GRF of ABL from PRE to Q2 was 55%.

Table 11. Average Max GRF as %BW Per Pig, Per Leg, Across Each Quarter

		IFL	AFL	IBL	ABL
PRE	Pig #1	103.15	97.68	67.61	64.52
	Pig #2	96.62	100.78	69.15	67.78
	Pig #3	76.85	79.29	57.73	58.16
	Pig #4	70.45	76.34	58.33	57.42
Q1	Pig #1	90.94	95.93	62.26	46.51
	Pig #2	92.17	81.23	69.53	36.90
	Pig #3	83.65	83.24	65.90	32.99
	Pig #4	72.99	74.27	58.30	35.08
Q2	Pig #1	84.51	89.14	65.76	38.29
	Pig #2	89.46	91.26	57.83	40.14
	Pig #3	77.38	76.44	60.82	18.55
	Pig #4	83.41	73.49	68.55	14.83

5.4.2.3 Push-Off Force

As described in 5.3.2 FORCE PLATE, the average magnitude of the push-off force was calculated using the Pythagorean theorem and measured in Newtons. This value for [IBL and

ABL] in Pigs #1-4 during PRE was [8.1,7.2], [7.8, 5.2], [6.9, 4.7], and [7.6, 5.2], respectively. This value for the same legs and the same pigs from the data collected during the last experiment for each pig was [4.7, 7.6], [8.3, 9.8], [8.6, 7.0], and [12.0, 0], respectively.

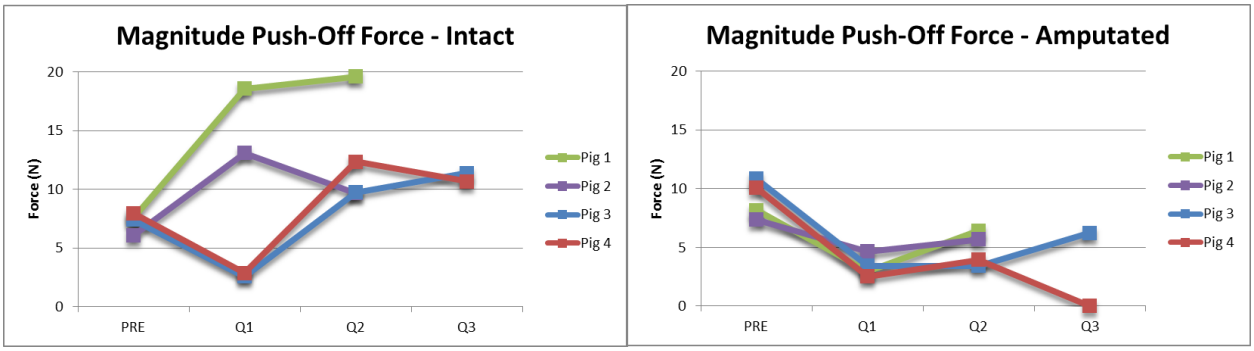

Figure 63. Magnitude of the push-off force for the intact hind leg and the amputated hind leg.

Additional force plate data can be found in A8. Gait Results.

5.5 DISCUSSION

5.5.1 DARTFISH DISCUSSION

5.5.1.1 Trials Collected

Q3 of the amputated limb is represented by only Pig #3 because Pig #4 stopped loading the prosthetic at week 8, so no data were collected on the amputated limb. With the exception of Q3, there was an approximately equal amount of data represented for each hind leg, giving an equal weight to the analyzed data from each hind limb across all animals.

Pig #2 had 64% more trials collected PRE than the other animals because there were a total of four experiments run on Pig #2, while there were only 3 experiments run on each of the other pigs. This difference made the pre-op data for Pig #2 less susceptible to outliers by

increasing its variance. More trials collected meant that there was more variation in the overall Pig #2 data, and the combined data for all animals PRE were more sensitive to the Pig #2 data. Pig #2's data was weighted more heavily than the other three pigs during PRE.

There was a decrease in the number of trials collected from PRE to Q1 because the animals would fatigue and be unable to continue. Q1 contained the four weeks immediately following surgery, so the animals had not had as much time to recover as they had at collection times in Q2 or Q3. This also explains why their velocity was slower overall in Q1 compared with PRE and is consistent with human activity following an amputation.[60]

For the ABL, the velocity was on average faster than the IBL during Q1 and Q2, which resulted in a shorter GC duration and an increased stride length. Due to the direct correlation between speed, stride length, and GC, the stance and swing times remained the same as % GC at any given velocity during normal gait of both hind legs.[61] While this was evident pre-op, that was not the trend that was seen post-op. Stance time as a %GC increased significantly over time for the IBL, and stance time as a %GC for the ABL decreased significantly over time. This relationship was consistent with limping in amputee patients.[62] Pain or discomfort caused by decreased flexibility, decreased range of motion, changes in leg length, or a loss of degrees of freedom, or any combination thereof could have caused this limp. This limp may have also been caused by the rigidity of the prosthetic or a mismatch in anatomical configuration. However, there is no way to determine which factor directly caused the abnormal gait pattern in this pilot study.

The velocity at which most data were collected pre-op was fairly evenly distributed across S2-S5. During Q1, the average velocity of all animals decreased, as was expected post-op. During Q2, Pig #1 achieved velocities similar to those collected during PRE, but did not reach the same maximum velocities during Q1. This was expected considering Q1 was the quarter immediately following surgery. The same is not true of Pig #2 and Pig #4 who were unable post-op to ever match their max pre-op velocities during data collections.5.5.1.1 Stride Length.

In normal gait of both humans and quadrupeds, the stride length increases with an increase in velocity as shown in studies performed by Jordan[61, 63] and DeCamp,[64] which was seen in the pre-op data collected. Due to the high amount of variance at each velocity for the ABL, there was no significant difference in stride length within each velocity over time. Additionally, the relatively large amount of change in standard deviation for the ABL from PRE to Q2 demonstrated that the stride lengths in PRE were much closer to the mean for each velocity than the stride lengths in Q2. There was no consistency of the stride length of the ABL during Q2, meaning that there was no repeatable distance the animals achieved for each stride length.

5.5.1.2 Gait Cycle

As expected, as velocity increased, GC, or the time from touchdown to touchdown and from liftoff to liftoff, decreased. This was supported by the data shown in Figure 47 as well as a study performed by Jordan on how velocity affects gait cycle parameters.[63] Additionally, with the exclusion of Q3, the change in average GC duration for the intact leg was almost equal to

that of the amputated leg. This supports the fact that the absolute values of the changes were directly proportional: if one leg was affected, so was the opposite leg. In other words, when one leg was amputated, the gait pattern of the remaining intact leg was altered equally and oppositely to compensate for the change.[65] This compensation was further demonstrated by a decrease in amputated stance time from PRE to Q2, while it increased for the intact limb across all speeds, showing an equal and opposite relationship. These findings were consistent with clinical results in human amputees. According to studies done by Bae et al.[65] on the dynamic analysis of amputee gait, this abnormal gait pattern represented a limp. Similarly, this abnormal gait pattern was consistent with DeCamp's assessment of lameness in dogs.[64] When the subject as more comfortable loading the intact limb, it had a much higher stance time than the amputated leg and looked like a limp.[64]

There was a strong correlation between duration of GC and both stride length and velocity from the data collected from both the intact and amputated legs as shown in Table 8 and Table 9, respectively. This correlation as consistent with the findings of Jordan et al.[61] Additionally, there was a significant negative correlation ($r \leq 0$) between duration of GC and both stride length and speed. As velocity increased, stride length increased, and duration of GC decreased. Oppositely, as velocity decreased, the stride length decreased, and the duration of GC increased. The greatest difference in duration of GC was found between S1 and S2 for both hind legs, and the least amount of change occurred between S4 and S5. Velocity 1.5-1.75 m/s is shown solely as evidence further of the trend in Figure 48. This trend was consistent with the findings of Jordan et al.[61]

There was no significant change over time in stride length or duration of GC within each velocity category for either hind leg. Additionally, while the amputation affected the velocity, stride length, and duration of GC; the amputation did not have any significant effect on the relationships between speed, stride length, and duration of GC.

5.5.1.3 Speed

Of the data collected, Pig #1and Pig #2 were more evenly distributed as far as velocity preference pre-op, but Pig #2 was slower than Pig #1 in Q2, never reaching a velocity above 1.75 m/s. Pig #3 seemed to prefer 1.0-1.25 m/s in 75% of the quarters, while Pig #4 seemed more comfortable with a slightly slower pace, not exceeding 1.0 m/s in over 76% of all trials collected from the ABL. No trials were collected on Pig #4's amputated leg in Q3 because he stopped loading after Q2. In Figure 53, each pig was thought to have preferred a different velocity throughout the study during data collection. Also, velocities exceeding 1.75 m/s were shown to be rare post-surgery, occurring in less than 1% of all trials collected post-op, and only occurring in Pig #1. This indicated that the amputation inhibited the maximum achievable velocity of Pig #1.

Pig #4 was the slowest of all the animals for both hind legs with over 65% of all his trials being no faster than 1.0 m/s. This may have been directly related to the fact that he stopped loading his ABL after week 5, but it is unclear whether or not velocity had any effect on healing. Additionally, this trend in velocity may have been considered normal for this particular pig considering the similarity to the trial distribution across velocities during PRE experiments.

While some trends in velocity preference were observed within each individual pig over time, there was no overall velocity trend exhibited across all four animals.

5.5.1.4 Prosthetic Angle

Extension of the amputated leg was a factor presented as a concern after the first two pigs were observed not fully extending toward the end of the study. Additionally, during dressing changes while the pig was unconscious, we noted tightened semitendinosus and semimembranosus muscles [Figure 64] in Pigs 3 and 4 in the last 3-4 weeks of the study. This unnatural muscle contracture kept the legs from being fully extended and resulted in Pig #4 not touching down with the prosthetic for the remainder of the study. The muscles in the figure are shown for both a pig and a human for the purpose of showing similarity.

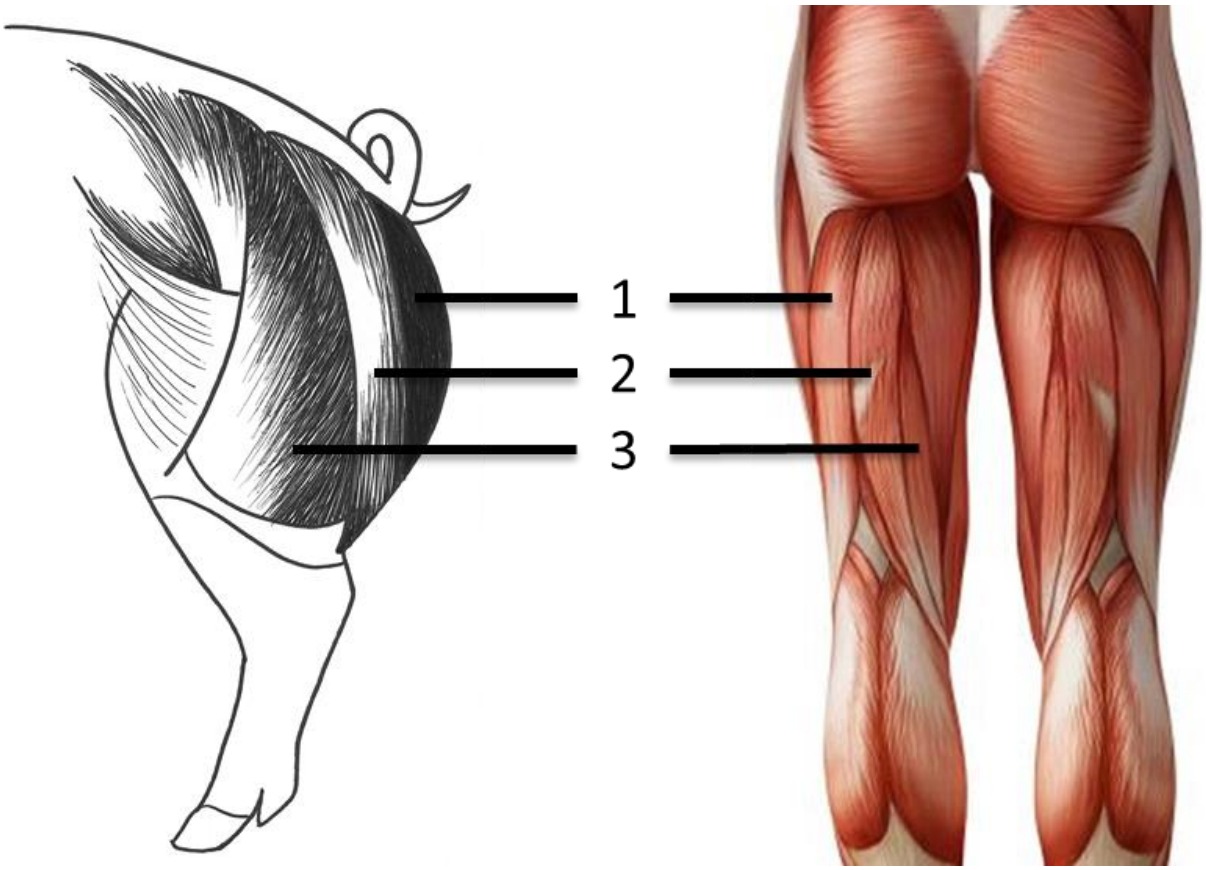

Figure 64. (1) Bicep femoris, (2) semitendinosus, and (3) semimembranosus muscles labeled in pigs (left) and in humans (right).

A trend observed in three of the four pigs was an overall decrease in maximum prosthetic angle post-surgery. This trend is strong evidence of contracture of the extensor muscles shown in Figure 64. Pig #4 was the only animal whose overall average prosthetic angle immediately post-surgery was less than the average prosthetic angle collected during the last experiment. However, Pig #4 was the only animal with data from week 1 immediately post-op. If the values taken from week 2 were compared with the last data set collected, the data would be consistent with the other three pigs in that an overall decrease would be seen in the max prosthetic angle. Pig #4 also stopped loading his prosthetic after 5 weeks post-surgery, therefore no data were collected on Pig #4's ABL after 5 weeks post-surgery.

Pig #3 is the only pig that showed an increase in max prosthetic angle. This increase occurred after week 6, which is the average healing time, and could be directly related to the healing of the wound. Additionally, 63.7% of all trials collected on Pig #3 during Q3 fell within S3, compared with only 28.6% of trials collected during Q2. An increase in velocity and an increase in prosthetic angle from Q2 to Q3 were consistent with the average healing time which would have occurred during Q2. Pigs #1 and #2 were sacrificed in Q2, and Pig #4 stopped loading his prosthetic during Q2, so no other pig had data collected on the ABL within Q3 for comparison.

The prosthetic angle range did not significantly change between Q1 and Q2 for all animals, showing that the min prosthetic angle decreased proportional to the decrease in max prosthetic angle. Although both the max and min prosthetic angle decreased over the course of the study in most of the animals, the muscle contracture did not affect the range of prosthetic angle. Only the amount of extension the animal could achieve with the amputated leg was affected as supported by a decrease in max prosthetic angle.

5.5.1.5 Example Gait

At 60 days post-op, Pig #4 was not loading the prosthetic, so the end of the prosthetic never actually touched the ground, as evident in Figure 57. Because of the lack of consistency of marker location, it is difficult to draw any conclusions from these figures. One thing to note would be that, even though the animal was not loading the amputated leg, there was still movement of the leg as though some attempt at ambulation had been made.

The lowest marker used on the ABL was at the bottom of the prosthetic (D) [Figure 42], while the lowest marker used on the same leg pre-surgery was located at the most distal point of the hoof (F) [Figure 41]. In Figure 56, marker F moved approximately .45 meters during the swing phase, while marker A, located at the hip joint, only moved .18 meters. Conversely, marker F didn't move at all during stance phase, and marker A moved .24 meters. Since the animal and its joints were moving the same distance overall, the distance the markers moved during the entire gait cycle should have been equivalent. However, that was not seen in this excerpt of gait cycle. This could be an indication of a lack of steady-state ambulation for this particular data set. The stride length shown in the pre-op gait data example was less than the average of stride length values of all animals in the slowest velocity category. However, for Pig #4, this slow velocity was comparable to the consistently slower velocities of Pig #4 compared with the other three animals.

Looking at the example gait post-amputation from Pig #4 in Figure 57, marker D was consistently located posterior to the x-location of marker A, while the pre-amputation data shown in Figure 56 demonstrates marker F alternating translational movement with marker A. This alternating movement of markers F and A pre-amputation was consistent with gait patterns in animals[60, 61, 63, 65, 66] where the limb is loaded during ambulation. However, this movement is not seen in Figure 57, which is what would be expected if that limb were not being loaded. When the animal extended the limb during swing pre-op, maker F was moved anterior to marker A, but post-op marker D remained posterior to marker A, showing a lack of extension of the prosthetic leg. This lack of extension was further evidence of muscle contracture.

Data from Pig #4 was chosen in order to better understand how the muscle contracture affected the ABL once the contracture had progressed to a point of inhibiting extension of the limb and preventing the animal from loading the prosthetic altogether. This data presented was collected from the only pig that stopped loading the prosthetic entirely and does not represent standard gait of any of the other three pigs post-surgery.

The amount of vertical distance the hip marker moved pre- versus post-op was approximately equal, while the knee marker vertical distance moved was greater post-op. This difference in knee distance traveled was further evidence of a limping motion as supported by a study published by DeCamp in 1997.[64] The distal-most point traveled a distance approximately equal pre- versus post-op, indicating that the animal was attempting to make the same motions of ambulation that he made pre-op, but he was unable due to the inhibited extension by the contracture of the extensor muscles shown in Figure 64.

5.5.2 FORCE PLATE DISCUSSION

5.5.2.1 Torque

For each data set collected from Pig #1, MZ was positive for the ABL and negative for the IBL. Positive torque represented a "screwing in" motion, while negative torque represented an "unscrewing" motion. This meant that the implant was unscrewing during gait, but it would be screwing in if it were in the opposite leg. This torque information further supported our decision to change legs.

5.5.2.2 Vertical Ground Reaction Force

Neither the GRF of the front two legs nor the GRF of the back two legs were significantly different pre-op which allowed for an accurate comparison of how the front and back legs changed to compensate for the amputation. The intact-side front leg and the intact-side back leg increased their loads in all animals to account for the decrease in support from the ABL post-surgery. The ABL percent weight distribution (%WD) decreased over time, while the IFL and IBL compensated. This was consistent across all animals over time.

The maximum %BW decreased in all legs for Pigs #1 and 2 from PRE to Q2. This trend was consistent with the decrease in speed: the slower the speed, the less force exerted during ambulation.[61, 63] However, the IFL and the IBL in Pigs #3 and 4 increased from PRE to Q2. This is consistent with abnormal gait characteristics in quadrupeds: when one limb is compromised, the opposite leg is unaffected while the adjacent legs compensate. [64, 65]

5.5.2.3 Push-Off Force

The magnitude of the push-off force is shown for all pigs across both hind legs in Figure 63. All pigs had an increased overall magnitude post-op for the intact leg and a decreased overall magnitude for the amputated leg. This was consistent with the amount of weight distributed between the hind legs post-surgery and the change in velocity pre- vs. post-surgery. Pigs #3 and 4 had a decreased magnitude push-off from PRE to Q1 for the IBL, which was consistent with the decrease in velocity and %WD to that leg. However, the push-off force of the IBL of Pigs #3 and 4 increased from PRE to both Q2 and Q3, which was consistent with the increased stance time and increased %WD to that leg.

All four animals had a decrease in magnitude of the push-off force for the ABL from PRE to all quarters post-op. Additionally, with the exclusion of Q3, each pig showed the same trend in the ABL from PRE to Q1 to Q2. This supported the findings of decreased speed, decreased loading, and decreased max prosthetic angle. This could be evidence of an irritated wound causing discomfort, pain, or a lack of wound healing.

5.6 ERROR

Repeatability, or test-retest reliability, was determined by Cronbach's alpha for all data collected using Dartfish. All Dartfish data were analyzed twice at different times no less than 8 weeks apart. The "CORREL" function in Excel was used to determine the internal consistency and repeatability of the analysis method using the video analysis software. Cronbach's alpha for each variable measured is shown in Table 12. GC Start Time, GC Transition, and Stride Length all had an $\alpha > .97$. None of the marker locations had an $\alpha \geq .9$. The average α of the marker locations was .65161. The higher the pairwise correlation, the more consistent the data as, and the less amount of human error was introduced.

Table 12. Internal Consistency - Cronbach's Alpha

Variable Measured	Cronbach's Alpha (α)
GC Start Time*	0.98484
GC Transition*	0.98729
GC End Time*	0.95801
Stride Length	0.97204
Prosthetic Angle	0.90113
Marker Locations: Average α of X and Y	
Hip Marker	0.42683
Knee Marker	0.51032
Posterior Calcaneus	0.86255
Proximal Metatarsals	0.68911
Distal Metatarsals	0.52416
Distal Point of Hoof	0.89671

* GC times are either Support Start Time; Support End Time/Swing Start Time; Swing End Time |or| Swing Start Time; Swing End Time/Support Start Time; Support End Time, depending on which phase came first.

There was a very high internal consistency (α) with stride length, and prosthetic angle, and all time variables. As anticipated, the error was introduced in uncertainty during determination of location of external markers.

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Chapter 6. Conclusions

The goals of this pilot study were to create a successful animal model for the TOI procedure, prevent infection, promote epithelialization at the skin-to-implant interface in an attempt to close the wound permanently, and to collect pre- and post-op gait data to analyze gait kinetics and kinematics.

6.1 SPECIFIC AIM I

The first specific aim of this project was to develop a protocol for and perform a hind leg amputation on four pigs, modeled after an above-the-knee amputation on humans. A surgical procedure replicating a human above-the-knee amputation was developed using cadavers and executed successfully on all four animals. During validation of the surgical procedure on Pig #1, four changes were made to the protocol for the remaining three pigs:

1. Morphine was used for spinal blocks for surgery instead of buprenorphine.
2. The residual limb was wrapped to the animal's body as shown in Figure 30 for the first two days post-op, and then the prosthetic was attached.
3. Holes were added at the proximal end of the implant in order to provide a more geometric fit for the bone cement to the implant.
4. The right back leg was amputated instead of the left back leg to prevent the implant from being unscrewed during ambulation throughout the study.

6.2 SPECIFIC AIM II

The second specific aim was to prevent infection while monitoring and promoting wound healing. Clinical infection was successfully prevented in all four pigs by rigorous wound

cleaning and dressing changes. Therefore, soap+H_2O was found to be a sufficient and effective method for cleaning the stoma. Observation of the wound over the course of the study showed a progression of healing in all four animals, but did not conclude with a seal of any kind. Upon visual inspection of the wound over the course of 11 weeks, there were signs of wound healing and new epithelial growth, though none that could be characterized as significant.

6.3 SPECIFIC AIM III

The third specific aim of this project was to collect and analyze both visual and numerical data on gait and weight distribution repeatedly both pre-op and post-op to determine the level of adaptation to the amputation as well as the potential impact the amputation had on the other joints.

Over the course of the study, each animal began to favor the intact limb as shown by increased support time compared with the decreased support time of the amputated limb. As velocity increased, swing time increased, providing evidence of limping. Across all pigs, the preferred velocity pre-surgery was inconclusive. However, the preferred velocity post-surgery was first 1.0-1.25 m/s and second .75-1.0 m/s for both hind legs. This suggested that the animals walked more slowly with three legs and a prosthetic, although there was no direct evidence relating velocity to wound healing.

The only pig with prosthetic angle data after 8 weeks was Pig #3, for an n=1. After 6 weeks, which is the average known healing time of humans, the max prosthetic angle had an upward trend. Although this population number is not large enough to draw any general

conclusions linking healing time to increase in flexibility, it does present reason to investigate this trend in future studies. Additionally, the muscle contracture was supported by the overall decrease in max prosthetic angle across all four pigs post-surgery. After consulting physical therapists, muscle contracture is common in amputees and can be prevented by active and passive stretching.

The information presented in the example gait graphs is for information only. Prior to this study, there was a lack of information on ambulation of a pig. No definitive conclusions can be drawn from this data alone.

The greatest changes in weight distribution were seen between opposite legs: amputated back leg and intact front leg. With a significant negative correlation, the intact-side front leg compensated the most for the decrease in weight-bearing of the amputated back leg. This suggests that, over an extended period of time, the intact front leg would undergo more wear than the other limbs, which would lead to more problems at the joint level due to the uneven distribution of weight.

The magnitude of the push-off force decreased with the amputated hind leg and increased with the intact hind leg. Weight distribution showed the same trend, as did speed. As velocity decreased, push-off force decreased, stride length decreased, and gait cycle time increased. An additional relationship that was realized post-surgery was that, as velocity decreased, prosthetic stance time increased, and prosthetic angle increased. The results of the gait study will allow us to adapt our protocol for future studies to optimize the efficiency and maximize the reliability and repeatability of this full animal study.

Chapter 7. Limitations and Future Work

There were several limitations faced during this study, the biggest of all being that it was a pilot study. There were a number of "uncharted" areas where the outcomes and expectations were a mystery. To start, the length of the study for each animal was a different length of time. This was necessary for investigational purposes, but it made comparisons of results difficult across pigs. Once the round of pilot studies has concluded and the optimal study length has been determined, the time frame will be much more consistent and the data more directly comparable.

Due to the nature of working with animals, this was the second largest limitation faced. The lack of control of the subjects made consistency more difficult to achieve. For instance, if you're a pig, you're most concerned with eating whatever you can find. So performing a gait study isn't exactly at the top of your list of priorities. Additionally, if you get tired and feel like lying down, you do – no matter where you happen to be. And if nobody can convince you that getting up for some food to collect a few more gait trials is going to be better than having your belly rubbed, you're not going anywhere. These are just a few examples of limitations we faced.

The study became more sophisticated with the second round of animals, but there are still a number of unknowns. The muscle contracture issue became obvious during the second round of pigs. The living situation of the first two animals allowed them more room to maneuver and resulted in a higher activity level, where the living conditions of the second two animals did not allow for as much activity. As a result, the muscle contracture could have been delayed in the first two animals because they were more active. However, there was no hard

evidence to support that hypothesis one way or another. Additionally, the second round of pigs was more dormant and spent a lot of their days lying down. This inactivity promoted muscle contracture. Since this is most commonly prevented with active and passive stretching, we propose developing a prosthetic with some kind of elastic band that attaches to the animal's harness so that the prosthetic is gently and passively being pulled forward. This way, when the pig is spending the majority of its days lying down, the leg will be stretching in extension and hopefully will delay, if not prevent altogether, muscle contracture.

For future studies, we believe that increased frequency of gait studies will not only be good for data collection, but also increase the activity of the animals. Another limitation was the wound cleaning: we had to sedate the animal with isoflurane gas every couple of days to clean the wound and change the bandages. Over time, each animal became somewhat adjusted to isoflurane, so it was more and more difficult to fully sedate the pig as the study continued.

There was only one force plate, it was not synced with the motion capture, and the room was not designed for force plate data collection. There was noise in the data, and there was no way to link up the gait data after the study was completed. Additionally, we were not given permission by IACUC to place any kind of adhesive marker on the pigs' skin in order to track the locations of different joints. This made analysis of angles very difficult with low consistency rate. For future studies, we would propose using a motion capture system that utilizes markers of some kind for increased accuracy of data.

Another limitation of this study was that data were not collected on both hind legs at the same time during one trial. Each successful trial collected data on only one of the hind legs. In order for a more accurate comparison of gait characteristics across left and right hind legs, future studies should collect data on both hind legs simultaneously. Also, the four animals used in this study came from two different litters: Pig #1 and Pig #2 were from the same litter, and Pig #3 and Pig #4 were from the same litter. This genetic difference may have explained the variation across pigs, but no direct conclusion could be drawn.

We believe this model can be successful and, if advanced rapidly, could lead to FDA approval in the US. The human procedure is life-changing, and we think it has the potential to positively impact a significant number of lives.

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APPENDIX

A1. Yucatan Miniature Swine Information

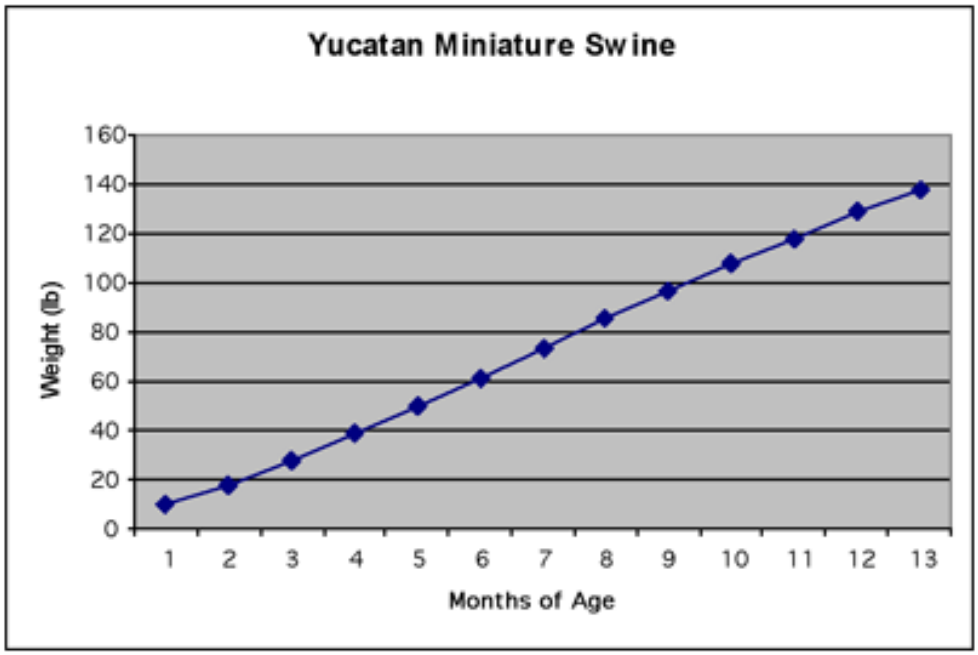


Figure 65. Yucatan miniature swine age-to-weight ratio.

sinclair bio-resources

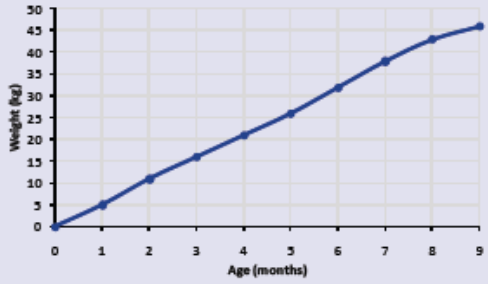
Miniature Swine Production

Yucatan Miniature Swine

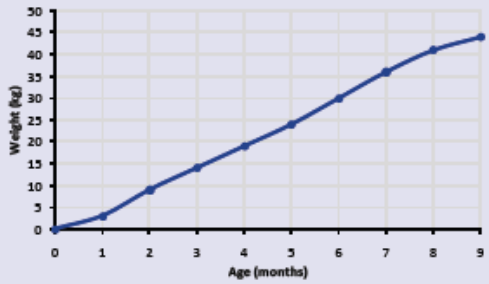
Yucatan Miniature Swine

Growth Curves

Male



Female



Background

- Domesticated miniature swine
- Sinclair acquired two colonies of Yucatan, one from Biotech Inc. and one from Charles River Laboratories (CRL).

Origin

- Both Yucatan colonies produced by Sinclair have the same origin.
- 25 animals imported into the United States in 1960. Developed at Colorado State University with funding from the Kroc Foundation.
- Sinclair assumed production of the Biotech colony in 1995 and the CRL colony in 2002.
- The Biotech colony is in Columbia, MO and the CRL colony is in Windham, ME.
- The colonies are closed and fully pedigreed.

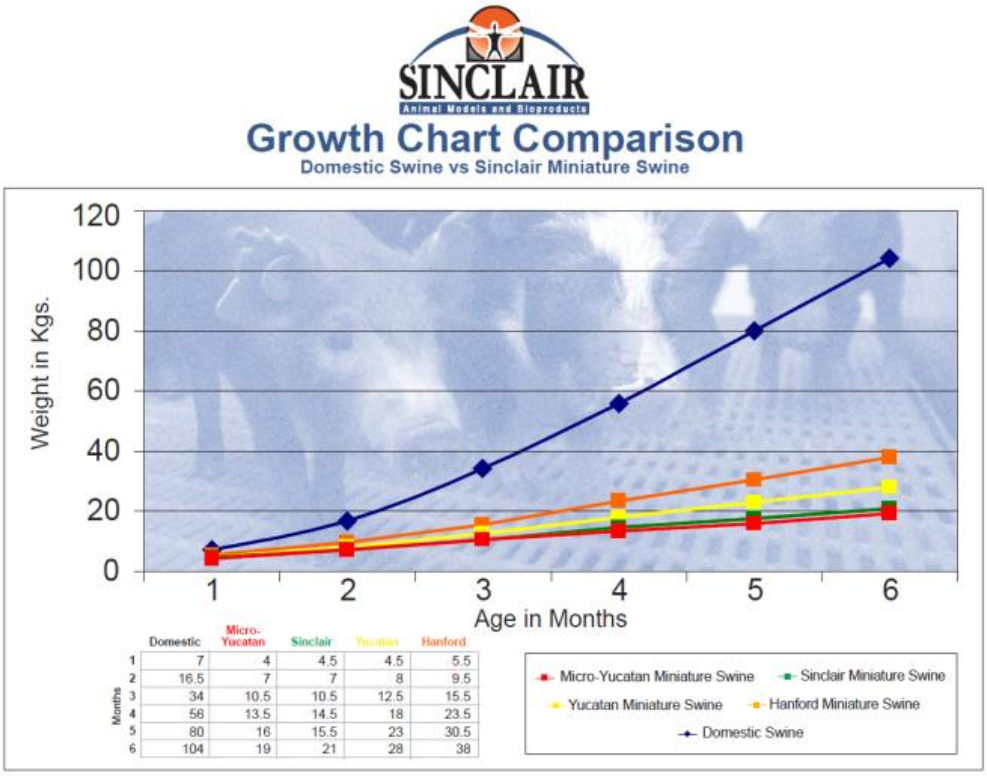
Characteristics

- Purpose-bred, socialized and vaccinated.
- Darkly pigmented skin with little or no hair.
- **White hairless** lineage for dermal studies.
- **VSD** lineage also available.

  DNA samples from the Biotech colony are banked and available for documentation and/or genetic studies.
- The Biotech colony is MHC haplotyped defined for transplantation studies (MHC genes of these lines have been cloned and sequenced for the SLA class I and class II and the characterizations of allele-specific monoclonal antibodies).
- Free from common domestic swine diseases; e.g. leptospirosis, brucellosis, pseudorabies, transmissible gastroenteritis, porcine reproductive respiratory syndrome, toxoplasmois, etc.

www.sinclairbioresources.com
info@sinclairbioresources.com
PO Box 658 Columbia, MO 65205
Tel: (573)387-4400 • Fax: (573)387-4404

Figure 66. Yucatan miniature swine fact sheet - Sinclair Bio-Resources.



Months	Domestic	Micro-Yucatan	Sinclair	Yucatan	Hanford
1	7	4	4.5	4.5	5.5
2	16.5	7	7	8	9.5
3	34	10.5	10.5	12.5	15.5
4	56	13.5	14.5	18	23.5
5	80	16	15.5	23	30.5
6	104	19	21	28	38

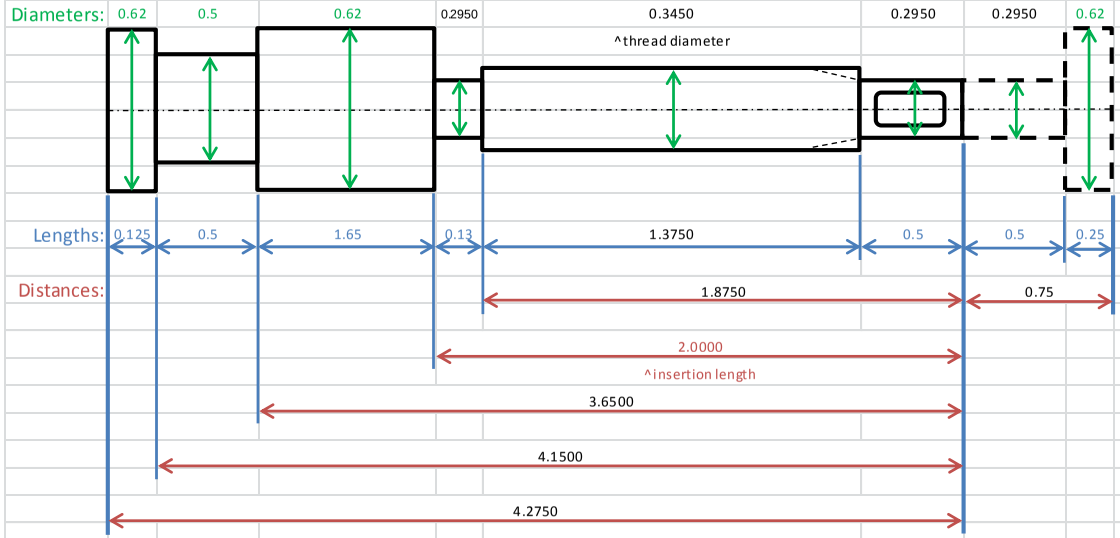
Figure 67. Different swine breed comparison in age-to-weight ratio.

A2. Implant Sizing Calculator

KUMC Orthopedic Research
Trans OI
Implant Sizing Calculator
7/25/2013

	Input (cm)	Conversion (in)
Insertion Length:	5.08	2.0000
Min. Periosteal Diameter:	1.46	0.5748
Min. Endosteal Diameter:	0.66	0.2598
50% P-E Difference Diam:	1.06	0.4173
Thread Interference:		0.0300

		5073	5074	
Total Length		14.35	16.64	cm
Min. Endosteal Diameter	ML	1	1.22	cm
	AP	**0.66**	**0.67**	cm
Min. Periosteal Diameter	ML	2.02	2.22	cm
	AP	**1.46**	**1.52**	cm



Transvese Cut Location		2		in
Insertion length		2.0000		in
Reamer Diameter	8mm	0.3150		in
Thread Interference	60%	0.0300		in
Thread Diamter		0.3450		in

Available Reamer Diameter (mm)	Conversion (in)	cm
7	0.2756	0.7
8	0.3150	0.8
9	0.3543	0.9
10	0.3937	1

Figure 68. Example implant sizing calculator.

A3. Silicon Cap Alternative

The European device uses a silicon cap [Figure 4, Figure 69] at the distal end of the stoma to hold any dressings used as well as aid in protecting the stoma. In adaptation to the animal model, we used a Poly Tank Float Ball [Figure 70], which we modified to fulfill our needs [Figure 71, Figure 72]. The Ball was cut along the blue dotted lines, and the part labeled "Black Cup" was modified further [Figure 71]. We drilled holes of varying sizes radially around the perimeter [Figure 72]. These holes were designed to allow the wound and stoma site to breathe. Due to the rough texture of the plastic, we added some clear plastic tubing which we cut axially and placed over the edge of the cup [Figure 74, Figure 73, Figure 75]. This provided a barrier between the skin and the cup to minimize irritation once the cup was implemented during wound dressings.

Figure 69. Human stoma with silicone cap and prosthetic attached.

Figure 70. Poly tank float ball.

Figure 71. Poly tank float ball: modifications.

Figure 72. Black cup with holes.

Figure 73. Tubing used to minimize abrasiveness of cup.

Figure 74. Black cup with tubing, profile view.

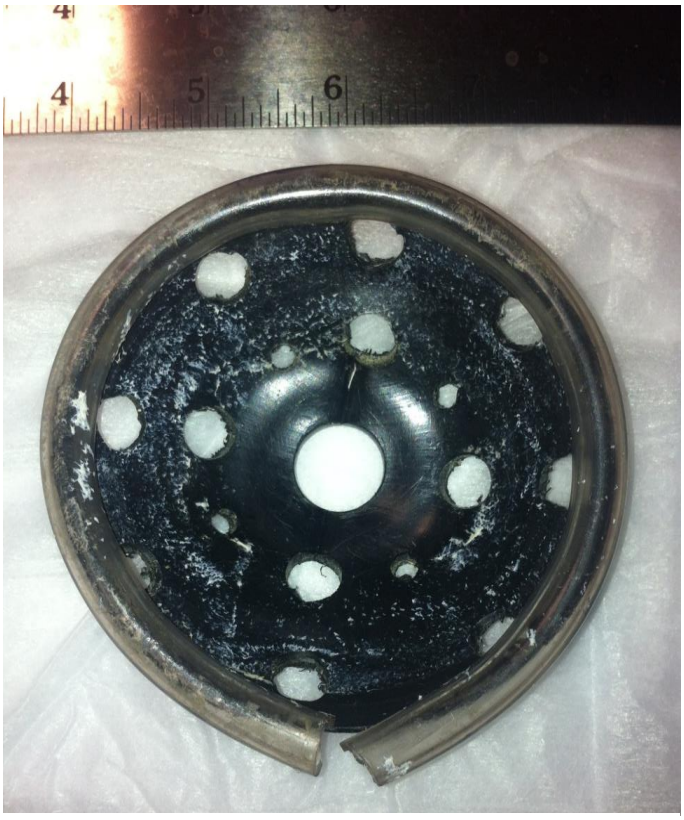


Figure 75. Black cup with tubing.

A4. Surgery

Table 13. IACUC-Approved Drugs Used Throughout the Study

Drug	Species	Class of drug	Hazardous (yes/no)	Dose	Route	Duration of treatment
Cephazoline	Porcine	Antibiotic	No	15 - 25 mg/kg	IV	Preop once
Atropine	Porcine	Anticholinergic Tranquilizer Sedative	No	0.02 – 0.05 mg/kg	IM	Preop once
Ketamine	Porcine	Dissociative agent (Schedule III)	No	15 - 25 mg/kg	IM	Preop and at x-ray
Xylazine	Porcine	Analgesic Sedative	No	2.0 mg/kg	IM	Preop (optional)
Isoflurane	Porcine	Anesthetic	No	1 – 2%	Inhalation	Intraop 1 to 1.5 hours
Flunixine Meglumine	Porcine	Analgesic NSAID	No	1 - 2.2 mg/kg	IM (neck)	1 to 5 days (every 24 hours)
Fentanyl	Porcine	Opioid Analgesic (Schedule II)	No	50 - 100 µg/hr	Transdermal patch	Up to 5 days (or more if needed)
Midazolam	Porcine	Sedative	No	100 - 500 mcg/kg	IM or IV	Daily: As required for procedural handling

Beuthanasia-D	Porcine	Barbiturate mixture (Schedule III)	No	150 mg/kg (.4 mL/kg)	IV	Once
Lidocaine	Porcine	local anesthetics	No	4.4 mg/kg	Epidural	Once (optional)
Bupivacaine	Porcine	local anesthetics	No	4.4 mg/kg	Epidural	Once (optional)
Morphine (preservative free)	Porcine	Local Anesthesia	No	.1 mg/kg	Epidural	Once (optional)
Buprenorphine	Porcine	Opioid Analgesic	No	.005-.01 mg/kg	SQ, IM	1-5 days, twice daily (Optional)
Carprofen	Porcine	NSAID Analgesic	No	2-4 mg/kg	PO, IM, SQ	1-5 days, twice daily (optional)
Omeprazole	Porcine	Proton pump inhibitor – treatment for gastric ulceration	No	.7 mg/kg	PO	Once daily, as needed
Famotadine	Porcine	H2 receptor antagonist – treatment for gastric ulceration	No	.5 mg/kg	IM	1-2 times per day, as needed

A5. Surgical Hole-Punch

During the second human surgical procedure, a stoma is created by cutting a circular incision through the skin and soft tissue for attachment of the transdermal coupler[5, 19] [Figure 76]. To simplify this meticulous process, we created a surgical hole-punch, subsequently referred to as "hole-punch". This hole-punch was made by sharpening one end of some stainless steel tubing [Figure 21]. It was used by twisting while pushing the sharpened end of the hole-punch into the soft tissue we wanted to cut [Figure 77]. This twisting motion would cut through each layer of soft tissue until the desired depth was reached.

Figure 76. Human stoma showing distal end of implant, immediately post-surgery.

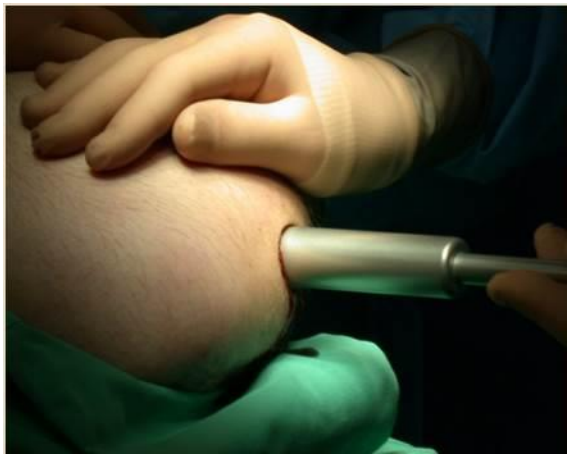


Figure 77. Human procedure, creating the stoma using a surgical punch.

A6. Histology

Figure 78 shows a sample of tissue retrieved with certain areas of interest labeled: healthy epithelial tissue, fibrin tissue, a breech in the skin's epidermal layer, and granulation tissue.

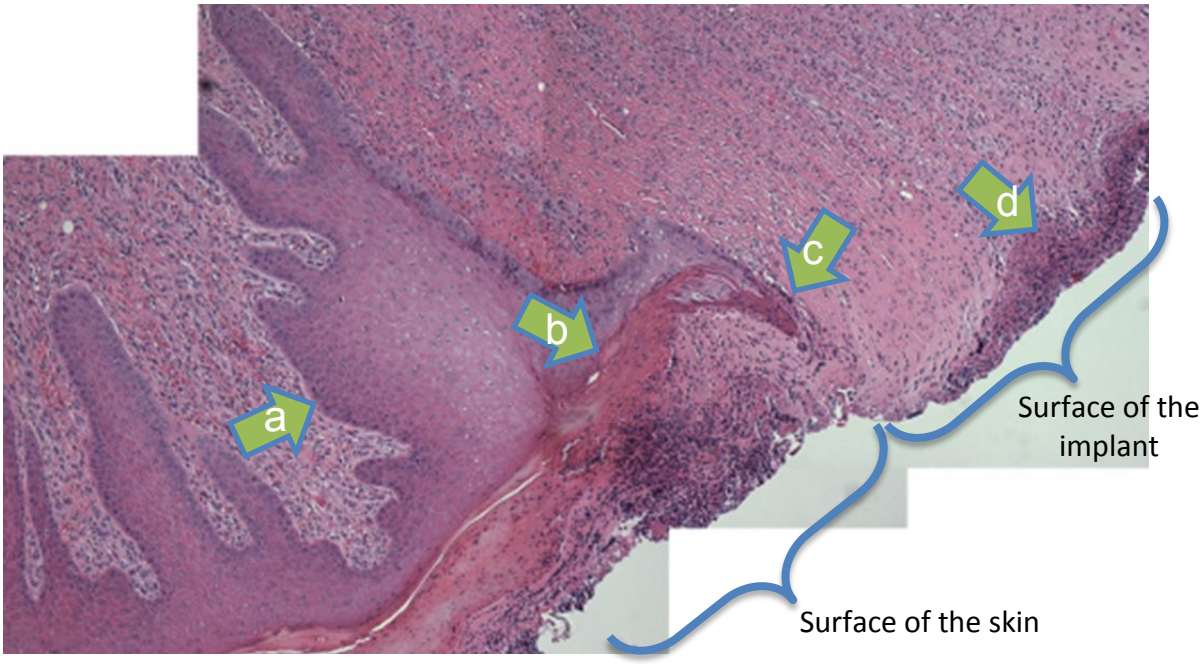
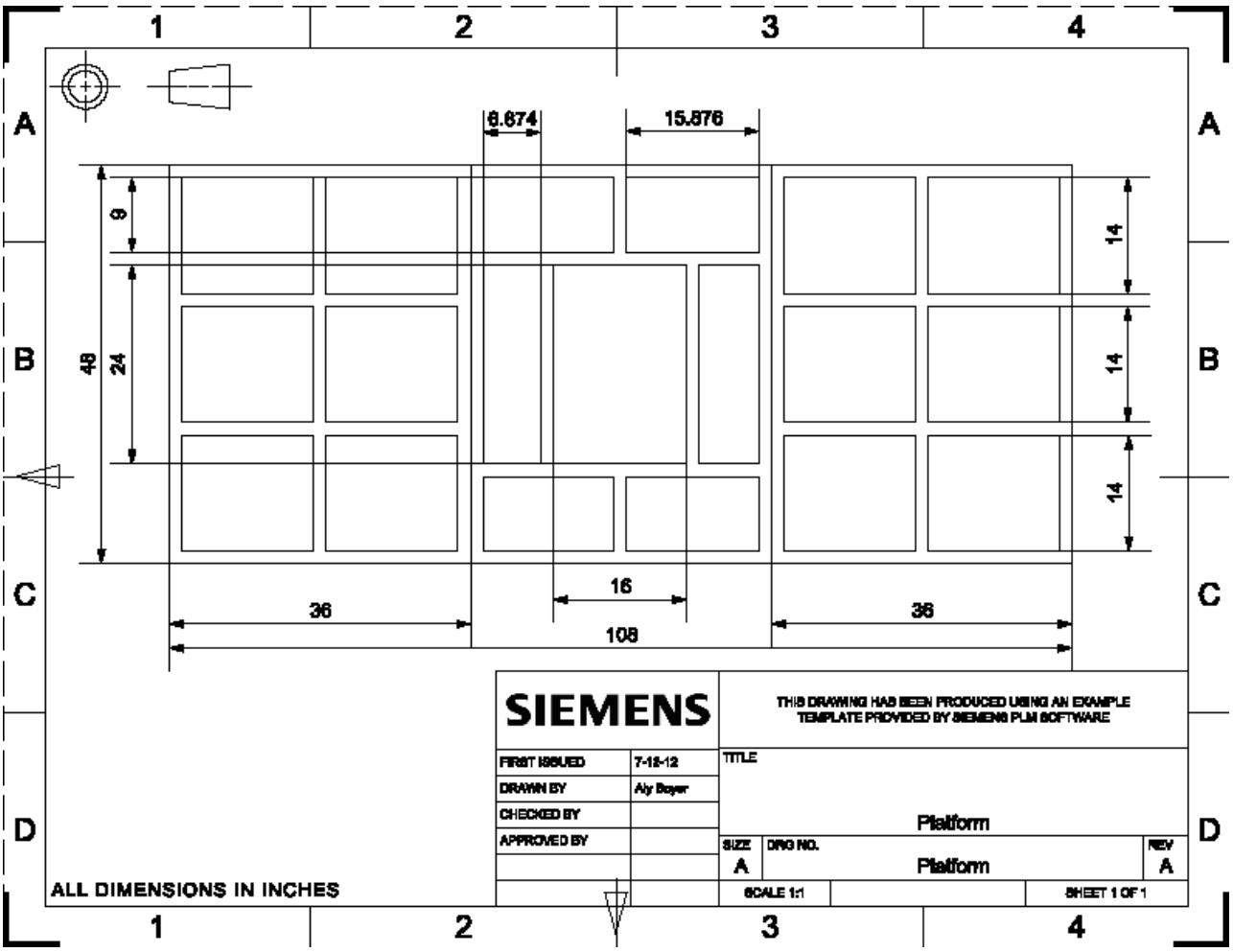


Figure 78. Histology slide of tissue from necropsy at 4x magnification. A) healthy epidermal tissue (skin); B) fibrin tissue (forms a scab for healing); C) breech in skin's epidermal layer, fibrin tissue growing to the surface instead of growing over to the wound near D; D) granulation tissue – developing skin layer, granulation tissue replaces the fibrin clot.

A7. Platform Drawing



A8. Gait Results

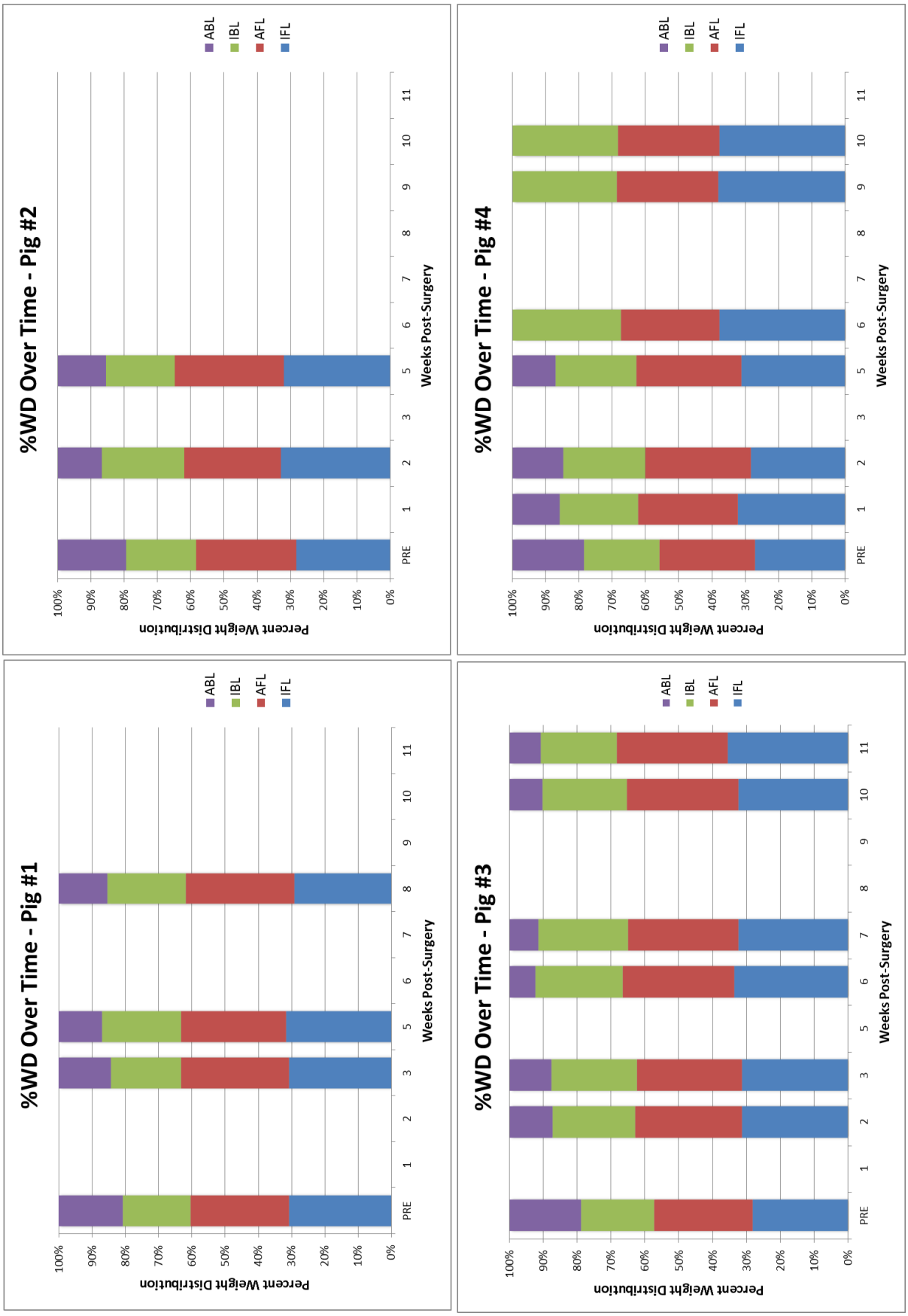


Figure 79. %WD over time for all pigs, normalized with all weeks shown.

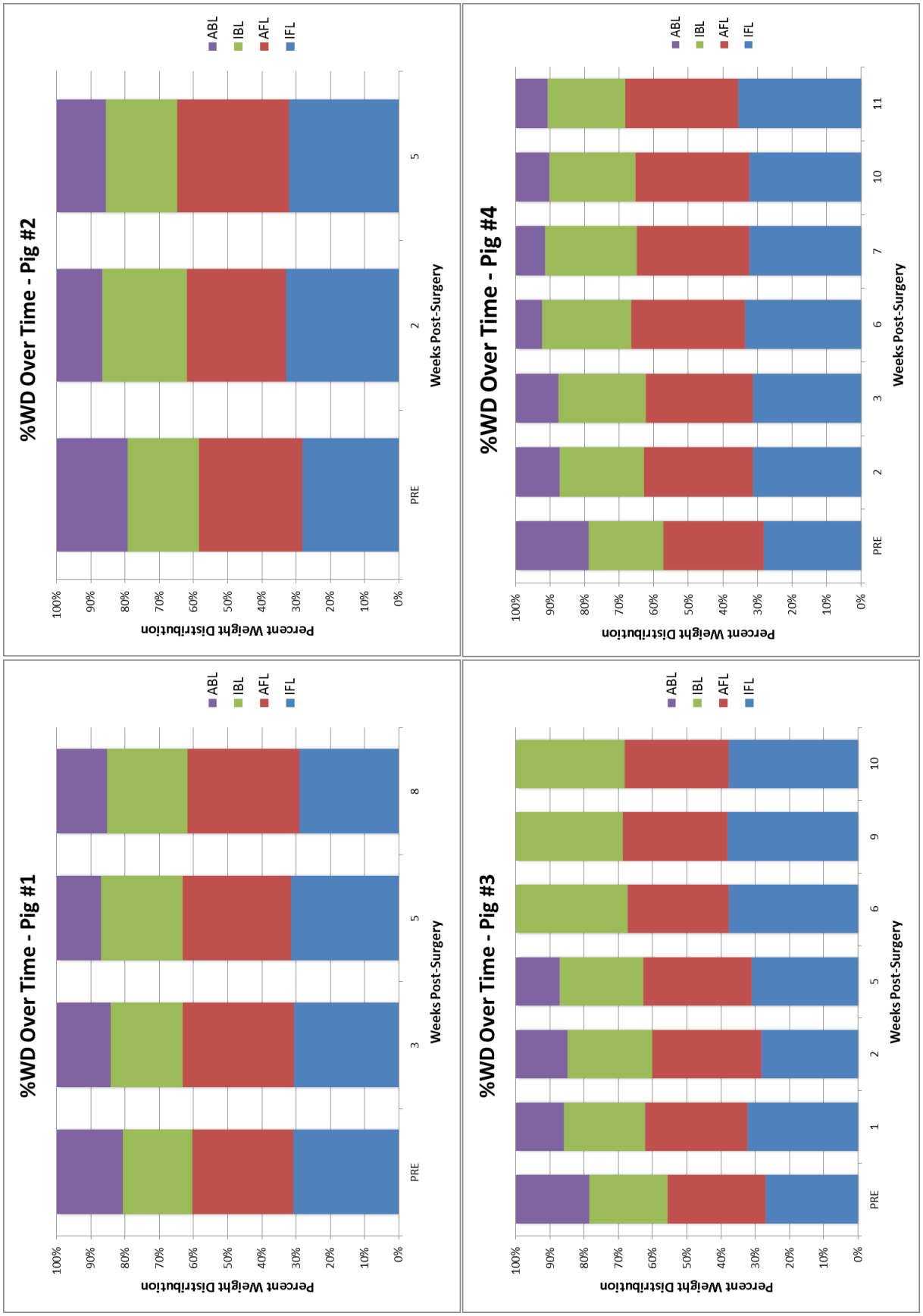


Figure 80. %WD over time, shown by week per pig.

Table 14. Max FZ, Average Per Experiment Pre-Op For Each Pig – Shown As %BW.

FRONT								BACK							
AMPUTATED SIDE				INTACT SIDE				AMPUTATED SIDE				INTACT SIDE			
Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4
77.37278	95.78982	72.13065	68.60193	91.18226	95.885	73.90189	65.18779	56.97901	66.89086	54.25039	52.72603	54.08275	68.91181	52.60811	52.68831
101.9433	103.8365	73.90865	66.91235	106.5647	98.2773	70.21815	60.91683	66.86254	71.36252	55.16609	52.44117	76.65178	74.31215	54.14847	51.59859
113.7307	110.4715	91.81993	93.492	111.7092	113.7827	86.43923	85.25356	69.70569	78.93074	65.05051	67.09798	72.09726	81.02907	66.43499	70.6934
	93.01339				78.52204				53.92952				52.35978		

Table 15. Max FZ, Average Per Experiment Post-Op For Each Pig – Shown As %BW.

		FRONT								BACK							
		AMPUTATED SIDE				INTACT SIDE				AMPUTATED SIDE				INTACT SIDE			
Week		Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4	Pig #1	Pig #2	Pig #3	Pig #4
	1				69.55397				75.25605				32.60771				54.95217
	2		81.22805	88.7103	78.99054		92.1709	88.49811	70.73161		36.89611	35.26569	37.55498		69.53086	68.8377	61.64666
	3	95.92704		77.77256		90.9379		78.80248		46.50614		30.70832		62.26295		62.95486	
	4																
	5	90.1256	91.25764		73.39437	90.4062	89.46325		72.5981	37.08519	40.14227		29.65893	68.00797	57.82981		56.45029
	6			79.42356	73.59332			81.19048	94.2127			18.27086	0			61.6108	80.64431
	7			73.45161				73.56388				18.82294				60.03846	
	8	88.16163				78.62362				39.49612				63.51031			
	9				76.62964				95.6658				0				77.94155
	10			80.98536	74.05662			80.34355	91.90151			23.54826	0			61.45321	76.78027
	11			78.37205				84.9516				21.76285				53.48613	

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