Title
Response of the mandible and the masseter muscle to distraction osteogenesis in the minipig (sus scrofa)

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Response of the Mandible and the Masseter Muscle to Distraction Osteogenesis in the Minipig (*Sus scrofa*)

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

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in the

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of the

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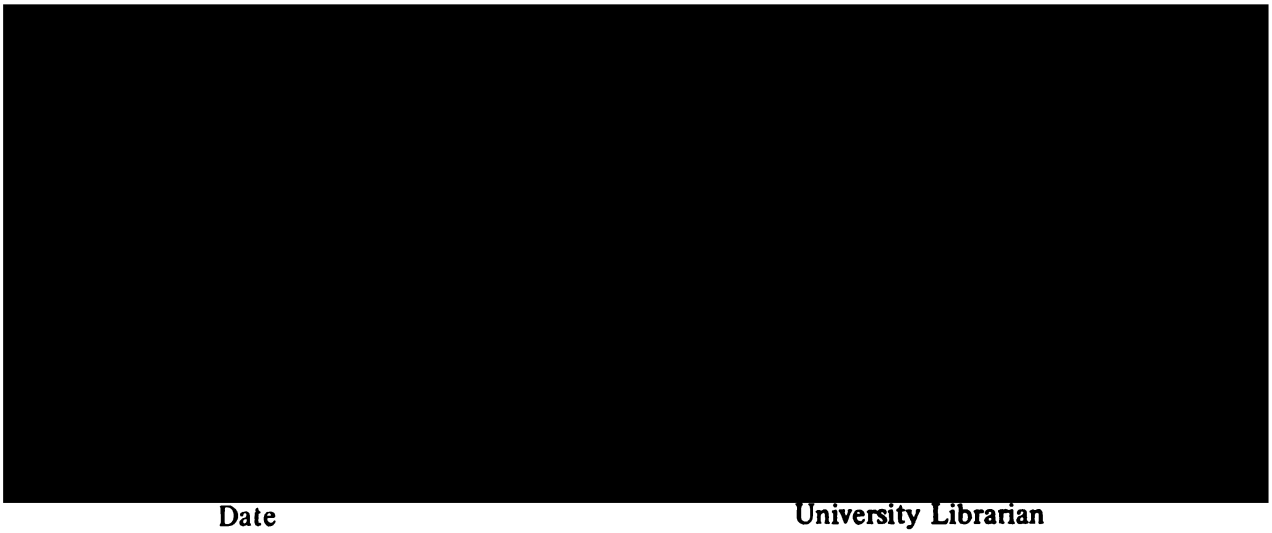

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Abstract

A pilot study was completed to determine the effects of mandibular distraction osteogenesis on the masseter muscle of the minipig (*Sus scrofa*). Of the six animals surgerized, two were successfully distracted and histologically analyzed (n=2). While the animal model appears appropriate, clinical evaluation and radiographic analyses suggest that a device "lever effect" was a significant factor contributing to a majority of the device failures. Analysis of the surgerized skulls revealed a 5.9 mm (17%) reduction of the ramus width on the experimental side ($P<0.02$), believed to have been caused by accelerated bony resorption from increased muscle tension resulting from distraction and scar formation. The procedure also produced unpredictable antero-posterior, vertical, and transverse mandibular changes as a result of difficult directional vector control when using a unidirectional device. Histologically, a 16% increase in muscle fiber number and 11% reduction in fiber cross-sectional area occurred on the experimental side ($P>0.05$). The greatest changes were observed in the posterior masseter, where a 37% increase in fiber number and 25% reduction in fiber area occurred ($P>0.05$). These results were consistent with the proposed hypothesis that fiber location and

orientation play a role in muscle response to chronic-stretch forces during distraction osteogenesis. Muscle fiber-type results were consistent with previously published results on masseter-muscle fiber composition in the growing pig ($P>0.05$).

Acknowledgements

This project could not have been completed without a tremendous amount of help from the countless bright and talented individuals to whom I am indebted. Special thanks to Dr. Hoffman, for allowing me to participate on this remarkable project; to Dr. Ferrari, for your guidance and expertise in working with the animals; to Dr. Vargervik, for your assistance in overcoming the tremendous logistical; to Dr. Miller, for your valuable insight into muscle fiber analysis and for your moral support; to Nilda, for your generous time sectioning the samples; to Ilse, for graciously allowing me to share your lab space; to Kingsley, for your effort with the histological images; to the wonderful veterinarian staff—Allison, Bronwyn, Cheryle, Cindy, Linda, Marsha, Mary, and Nina—for your love of animals and your help with the dozens of x-rays; to Lydia, for your help coordinating the surgeries and numerous emergencies with Bill; to the surgical staff—Ron, Chaigne, Gabriela, Percy, and Serena—for your flexibility and invaluable assistance; to the vet support staff—Chris and Alex—great job with the cages; to Stuart, for your patience with the statistical analyses, especially when n dwindled to 2; to Trang, for pointing me in the right direction with the histology; to Jane,

for your helpful histochemical insights; to Dr. Kapila, for allowing me to use your imaging equipment and lab space; to Midori, for teaching me how to properly stain the fibers; to Lisa, for a great job with the skulls; to Matt, for not taking on this project yourself; to Cindy, for your advice on solution preparation; to my co-residents, for suffering through 3 years of seemingly-endless discourses on this subject; and to my friends and family, for your love and support which made this entire effort worthwhile. This project was supported by a Synthes Maxillofacial - Bernd Spiessl Research Grant Award from the Maxillofacial Surgeons Foundation. We gratefully acknowledge the generous support of Howmedica for their contribution of materials for this study.

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A. Background

Distraction osteogenesis (also known as callostasis or callus stretching) is the process by which new bone is intentionally generated through the controlled, gradual, incremental separation of the healing callus following a corticotomy or osteotomy. This procedure can be performed without the need for additional bone transplantation. First described by Codivilla in 1905,[8] the technique was not popularized until the 1950's, when Ilizarov was able to demonstrate repeatable success distracting endochondral long bones of the upper and lower extremities to produce rather dramatic results.[27-29]

The original principles of distraction developed by Ilizarov (which are still debated) include the following: 1) *corticotomy* of the distraction site made to preserve blood supply to the periosteum and medullary canal; 2) stabilization of the bone for a *latency* period of five days following placement of the distraction device; 3) *distraction* performed at a rate of 1 mm/day and a rhythm of 0.25 mm/6h; and 4) fixation of the segments for a *consolidation* period equal to the distraction period once the desired length has been achieved.

Distraction osteogenesis has the potential to profoundly impact the correction of craniofacial deformities due to the fact that in many instances, skeletal correction through conventional surgical means does not produce an adequate amount of bone lengthening. Use of distraction osteogenesis for mandibular lengthening was first reported by Snyder in 1973 using a dog model.[55] In humans, bilateral treatment of Nager's syndrome (bilateral craniofacial microsomia) using distraction osteogenesis was first reported by McCarthy in 1992.[40] Since then, the technique has been modified and used to treat not only craniofacial microsomia, but also mandibular hypoplasia,[51] midface deficiency,[50] and Treacher-Collins syndrome.[45] Theoretically, this procedure could be used to lengthen any bone to which a distraction device could be attached and osteotomized. Numerous case reports and studies have been published on the use of distraction osteogenesis in the human mandible (Table 1).[8]

To date, most scientific findings have centered on bone histology[5,30,31,38] and surgical technique.[42,53] Much less is known about the effect of the procedure on the surrounding soft tissue. Studies on distracted rabbit tibias suggest that the periosteum slides over the bony cortex as the segments separate,[64] which is likely similar to the

Author	Year	Procedure	N	Bone division	Latency	Rate and Rhythm	Distraction	Consolidation	Problems and Complications
Guerrero	1990	Mandibular widening	10	Vertical symphyseal osteotomy	0 days	3 mm acutely, then 0.25 mm 1x/d	4-6.5 mm 4-14 d	12 weeks	NR
McCarthy *et al*	1992	Unilateral and bilateral mandibular lengthening	4	Oblique angle osteotomy	7 days	0.5 mm 2x/d	18-24 mm 20 d	8-10 weeks	Pin tract scars, loose and infected pins, relapse
Perrott *et al*	1993	Mandibular widening	1	Periosteal preserving osteotomy	7 days	0.33 mm 1x/d	10 mm 30 d	0 weeks - replaced by iliac bone graft and miniplate	Infected pins, mild discomfort, disproportional movement of inferior and superior cortices
Takota *et al*	1993	Unilateral mandibular lengthening	4	Corticotomy	14 days	0.36 mm 2x/d	17-22 mm 28-35 d	10-12 weeks - activator used for additional 6-12 months for retention	Pin tract scars, loose and infected pins, relapse
Habal	1994	Unilateral mandibular lengthening	1	Periosteal preserving subtotal corticotomy	7 days	0.5 mm 2x/d	NR	4 weeks	Pin tract scars, loose and infected pins, relapse
Havlik and Bartlett	1994	Bilateral mandibular lengthening	1	Osteotomy through previous bone graft	14 days	0.25-0.5 mm x/d	30 mm 46 d	0 weeks - replaced by iliac bone graft and miniplate	Loose and infected pins, device instability
McCarthy *et al*	1994	Unilateral and bilateral mandibular lengthening	15	Osteotomy	7 days	0.5 mm 2x/d	18-36 mm 18-36 d	8 weeks	Pin tract scars, dentigerious cyst secondary to pin placement, ankylosis of coronoid process/zygoma
Moore *et al*	1994	Bilateral mandibular lengthening	1	Corticotomy	5 days	1 mm 1x/d	20 mm 20 d	6 weeks	NR
Guerrero *et al*	1995	Bilateral mandibular lengthening or widening	20	Vertical symphyseal, ramus, or midbody osteotomy	10 days	2 mm acutely, then 1 mm 2x/d	3-10 mm 3-10 d	12 weeks	NR
Klein and Howaldt	1995	Unilateral and bilateral mandibular lengthening	9	Oblique ramus corticotomy	5 days	1 mm 1x/d	15-25 mm 15-25 d	9 weeks	Pin tract scars, loose fixation pins, facial nerve motor deficit
Kocabalkan *et al*	1995	Bilateral mandibular	1	Oblique angle corticotomy	5 days	0.25 mm 4x/d	18 mm 18d	6 weeks	TMJ pain, 2nd distraction procedure required
Molina and Ortiz-Monasterio	1995	Unilateral and bilateral mandibular lengthening	106	Oblique angle corticotomy or double level ramus/corpus osteotomy	4 days	0.25 mm 4x/d	12-29 mm 12-29 d	6-8 weeks	TMJ pain, occlusal interferences, loose and infected pins
Pensler *et al*	1995	Unilateral and bilateral mandibular lengthening	9	Oblique ramus or vertical midbody corticotomy	1 day	0.25 mm 4x/d	13-23 mm 16-40 d	3-7 weeks	Device interference with mastoid process
Rachmiel *et al*	1995	Unilateral and bilateral mandibular lengthening	3	Horizontal ramus or oblique angle corticotomy	7 days	1 mm 1x/d	21 mm 21 d	7 weeks	NR
Guyette *et al*	1996	Unilateral mandibular lengthening	2	Mandibular osteotomy	NR	NR	35-45 mm 40-50 d	NR	Unilateral openbite and crossbite, velopharyngeal inadequacy
Klein and Howaldt	1996	Unilateral and bilateral mandibular lengthening	18	Single or double level ramus/corpus osteotomy	4-5 days	1 mm 1x/d per osteotomy level	7-50 mm 7-50 d	7-9 weeks	Pin tract scars, loose pins, TMJ pain, trismus, premature consolidation, facial nerve motor deficit
Corcoran *et al*	1997	Unilateral mandibular lengthening	28	Oblique angle osteotomy through host bone or rib graft	7 days	0.5 mm 2x/d	9-25 mm 13-56 d	4-7 weeks	Pin tract scars and infections, device failure, fibrous pseudoarthrosis, transient neuropraxia
Polley and Figueroa	1997	Unilateral mandibular lengthening	2	Oblique angle osteotomy	7 days	1 mm 1x/d	30-35 mm 30-35 d	NR	Unilateral crossbite
Wangerin and Gropp	1997	Unilateral and bilateral mandibular lengthening	15	Horizontal ramus osteotomy	6 days	1mm 1x/d	7-25 mm 7-25 d	6 weeks	Transient loss of mandibular nerve sensation
Razdolsky *et al*	1998	Unilateral and bilateral mandibular lengthening	43	Midbody osteotomy	3-5 days	0.25 mm 4x/d	10-23 mm 10-23 d	3-7 weeks	NR

Table 1: Recently published reports on mandibular distraction ostegenesis

way the periosteum migrates during growth.[12,24,39,63] Work on rabbit-leg muscle by Simpson indicates that soft-tissue stretching at rates greater than 0.4 mm/day results in histological abnormalities, including necrosis at higher rates of distraction.[54] On the other hand, if the distraction rate is too slow, premature bony fusion occurs. Whether a clinical equilibrium exists that is healthy for the tissue but also effective for callus stretching is not known.

In the mandible, the effect of ramus distraction on the surrounding soft tissue may be especially relevant due the position of the masseter and its role as a primary jaw closer during chewing. Yet. little is known about the masseter's response to mandibular distraction. In the leg, distraction forces are parallel to the muscle fibers, whereas in the jaw, distraction forces may vary depending on the orientation of the muscle fibers. In the pig, rostral (anterior) fibers of the masseter orient more perpendicularly to the occlusal plane whereas dorsocaudal (posterior) fibers are more parallel.[23] In other words, if the mandibular ramus is distracted vertically, anterior fibers should experience parallel tensile forces, and posterior fibers fiber-separating perpendicular forces.

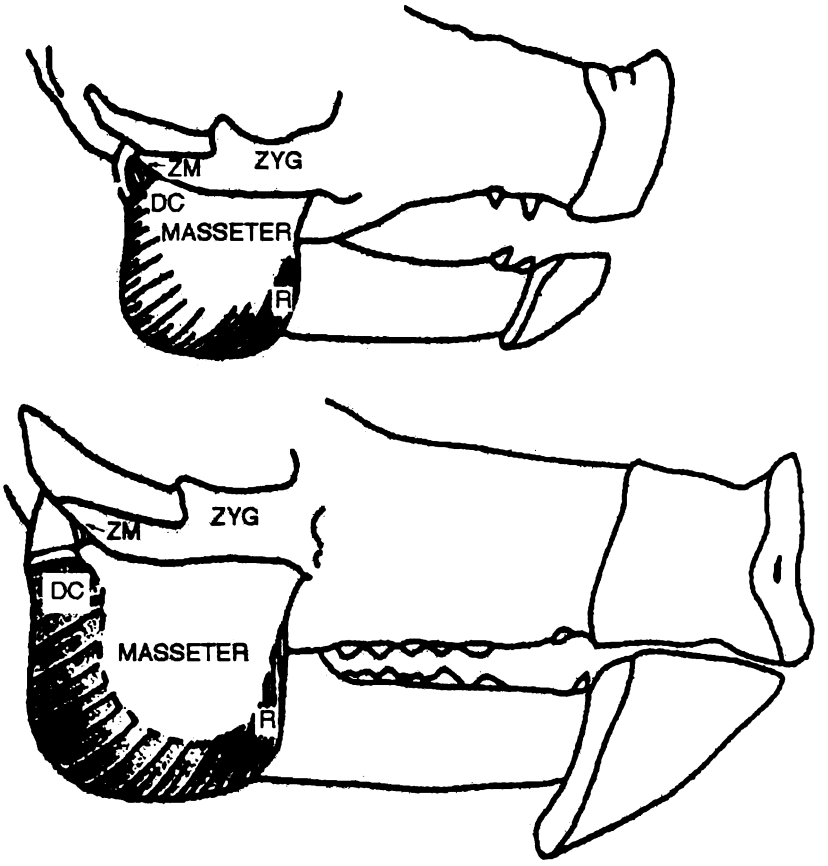


Figure 1 (Modified from Herring[23]): Orientation of masseteric muscle fibers in the juvenile and adult minipig. Anterior rostral fibers (R) are oriented more parallel to the ramus, whereas posterior dorsocaudal fibers (DC) are oriented more perpendicular to the ramus.

How then, does the direction of force affect the muscle fibers? Fisher has reported that fibers subjected to parallel stretch forces from distraction undergo hypertrophy, whereas fibers subjected to perpendicular forces undergo atrophy and decreased protein synthesis.[14] Studies on anterior latissimus dorsi (ALD) muscle in the Japanese quail show that chronic stretch leads to muscle fiber proliferation and stretch-induced fiber enlargement.[1] Given the

chronic-stretch nature of distraction osteogenesis, the masseter is hypothesized to undergo a similar type of proliferative response. A proliferative response would manifest itself as an increase in fiber density (number of fibers per unit area) from new fiber formation. Depending on the orientation of the muscle fibers relative to the position of the distractor device, one would expect to find hypertrophy in parallel fibers, and atrophy in perpendicular fibers. Hypertrophy would be seen as an increase in average fiber cross-sectional area, whereas atrophy would be seen as a decrease in fiber cross-sectional area. Furthermore, muscle capillary density (the average number of capillaries per muscle fiber) should increase with fiber proliferation/hypertrophy and decrease with fiber atrophy since capillary proliferation is one of the first biological adaptations to increased skeletal muscle use. In fact, capillary proliferation is observable even before changes are detectable in the oxidative enzymes of muscle fibers.[33]

Unknown is how the composition of muscle fiber type changes with distraction, if at all. The classification according to Brooke and Kaiser categorizes muscle fibers as either type I, IIA, IIB, or undifferentiated IIC.[5] Type I fibers (also known as slow β) are high in

oxidative enzymes and low in phosphorylase and ATPase, with the reverse being true for type II fibers (also known as fast α). In addition, Suzuki and Cassens have described the presence of subtypes of myofibers in porcine muscles, termed "intermediate (IM) fibers" as a whole.[57] In the growing pig masseter, type II fibers are predominant, but the percentage decreases with age. During growth, type II fibers are believed to convert through the intermediate fiber stage into type I fibers.[58]

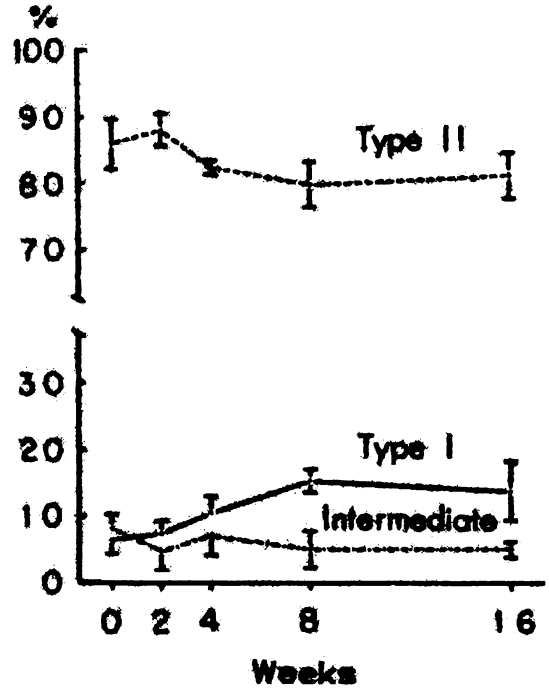


Figure 2: Normal fiber type distribution in the masseter of the growing pig (from Suzuki[58]).

Proliferative responses in the masseter may or may not alter the overall fiber-type percentages of a muscle. Carlson reported no

significant changes to anterior digastric muscle fiber composition following mandibular advancement surgery in juvenile monkeys (parallel forces).[7] Whether perpendicular stretching alters the percentage of fiber composition is not known.

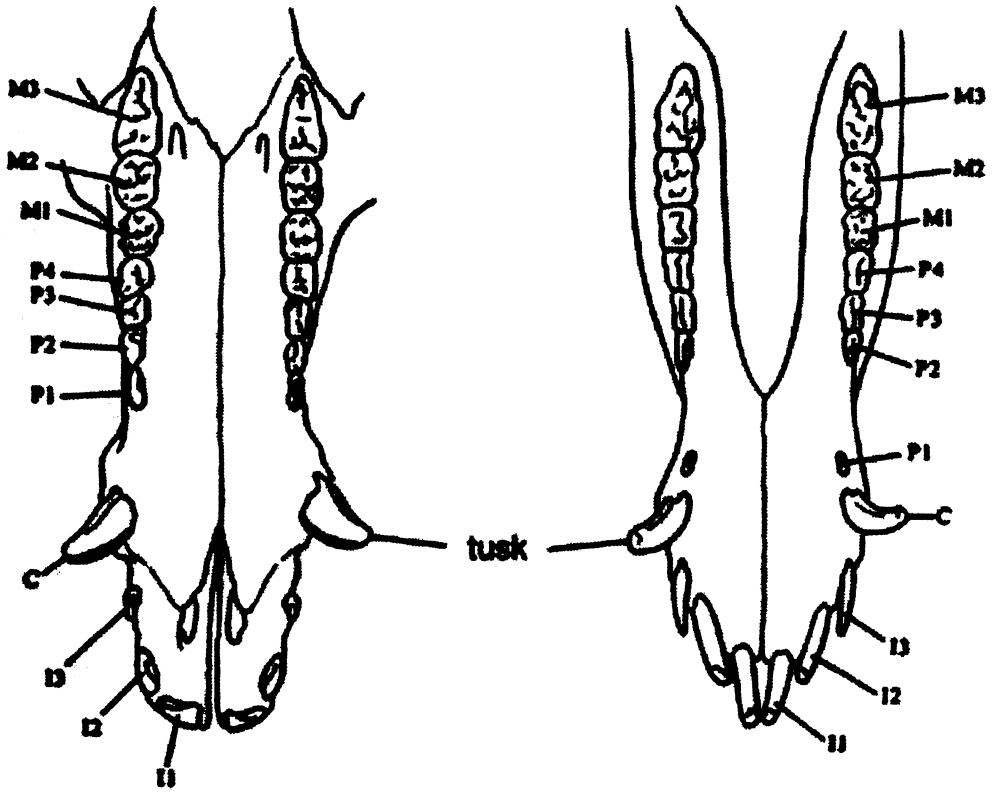
B. Specific Aims

The goal of this project was to demonstrate that the masseter of the minipig, when chronically stretched through distraction, would exhibit proliferative hypertrophy in regions where muscle fibers are parallel to stretch forces, and proliferative atrophy in areas where muscle fibers are perpendicular to stretch forces. The study is clinically relevant since any skeletal benefit derived from distraction may come at the expense of permanent, unavoidable changes to the form and function of the surrounding soft tissue. The effect of distraction on fiber-type composition was also investigated.

This project also intended to validate the minipig as an appropriate animal model for studying the effects of mandibular distraction osteogenesis on bone and surrounding soft tissue.

C. Experimental Design

The mandible of the female Yucatan minipig (*Sus scrofa*) was chosen as the site for surgical placement of a unilateral extraoral distractor. The minipig was chosen because (1) they have a predictable and accelerated rate of bone turnover and regeneration; (2) there are marked similarities between porcine mastication and human mastication; (3) pigs have an omnivorous diet comparable to humans; (4) the tooth size and dental development of pigs is comparable to that of humans (see Figure 3); (5) the embryology of the pig is well-known and resembles that of humans; (6) the miniature pig is available at a reasonable cost; (7) multiple animals from the same litter can be obtained easily; and (8) it is well established that pigs are amenable to anesthesia and surgery, with very predictable results and minimal preoperative and postoperative morbidity.[26] An animal model was used because the hypothesis could not be tested through cell culture or computer simulation.



Pig - aging teeth	Eruption
DI3	Before birth
Dc	Before birth
Remaining deciduous teeth	4 days - 7 wk.
Permanent	
P1	5 mo.
M1	4-6 mo.
Remaining permanent teeth	8-20 mo.

Figure 3: Dentition of the pig and approximate eruption dates

Female animals were selected because they are easier to manage. A unilateral extraoral device was chosen for its ease of

placement and activation. The number of animals required for sufficient statistical power was determined to be 6, and by using the contralateral side as the control, the total number of animals required for the study was reduced by half. Growing animals were chosen instead of adults, since distraction osteogenesis is currently being used to treat mostly developing children suffering from craniofacial disorders. In addition, growing minipigs are easier and less expensive to manage and care for than adult pigs are.

The device was oriented to separate the mandibular ramus primarily in the vertical direction. The parameters of the distraction procedure were largely adapted from Ilizarov's basic principles, with the primary difference being the use of a complete osteotomy instead of a corticotomy. A distraction rate of 1 mm/day was used, with a rhythm of 1 activation/day instead of 0.25 mm/6h for convenience. A latency period of 3 days was used instead of 5 days, since growing pigs develop much more rapidly than humans do. A consolidation period equal to the distraction period was used (as recommended).

Fifteen millimeters of total distraction length was chosen, corresponding to approximately 10% of the animal's mandibular ramus height. Weekly radiographs were taken to visualize the amount of

distraction achieved. Metal implant markers[3] and embedded silver-particle matrix placed at the time of surgery[12,16] were used to radiographically monitor bony separation and periosteal migration.

Because of the various fiber orientations of the masseter and the presence of multiple muscle compartments,[23] muscle biopsies were taken from different regions of the masseter—namely, the anterior, posterior, superficial, and deep regions on both control and experimental sides, similar to the manner described by Strohm.[56] While these regions do not represent all the compartments, they do represent a wide sampling. Superficial fibers were sampled in additional to deep fibers, since type II fibers can be more abundant in the superficial regions of certain muscles, necessitating the need to compare fibers sampled from similar regions.[13]

The muscle samples were characterized through comparative histochemical analysis of cross-sectional area,[25,56-58] fiber number,[1,2,15,61] fiber type,[4,5,19,60] and capillary density.[32,33] The mean and standard deviations of the cross-sectional area, fiber number, percent composition, and capillary density were determined for the left and right masseter muscles. The proportion of fiber types, their cross-sectional area, and capillary density were compared against the

same muscle site between the left and right side using a two-tailed, paired t-test. The level of significance was established at $P<0.05$. The same measures (*e.g.,* cross-sectional area) were also compared against sites in the same muscle using a single factorial ANOVA to evaluate if different regions of the same muscle were different. Spearman correlation coefficients were evaluated between fiber composition and capillary density.

D. Experimental Methods

D.1 Subjects

Six female adolescent Yucatan minipigs (*Sus scrofa*) were used in this experiment (Charles River Labs, Windham ME). All study animals were treated according to the ethical guidelines set forth by the University of California Committee on Animal Research. Animals were ordered and surgerized in pairs (unless otherwise noted) based on the amount of veterinary support required for adequate post-operative care. Upon arrival from the vendor, the animals were allowed to

recover for one week. They were fasted for at least 12 h prior to surgery. The animals were 5 to 10 weeks old at the time of surgery.

D.2 Surgical procedures

At surgery, the pigs were anesthetized with ketamine HCl (20 mg/kg IM; Ft. Dodge Animal Health, Ft. Dodge, IA), xylazine (2 mg/kg IM; Butler, Columbus OH), and atropine (0.04 mg/kg IM; Butler, Columbus OH), intubated, and anesthesia maintained with 1 to 3 percent isoflurane (Phoenix Pharmaceuticals; St. Joseph MO). Cefazolin (11 mg/kg IV; Marsam Pharmaceuticals, Cherry Hill NJ) was also administered pre-operatively. Prior to surgery, the experimental side was randomly determined, and 3 cc of 2% lidocaine (with 1:100,000 epinephrine; Astra, Westborough MA) was administered subcutaneously around the surgical site. A 1" incision was made along the middle one-third posterior border of the mandible. The masseter muscle was then carefully dissected and the periosteum elevated approximately where the ramus would be osteotomized, without stripping any muscle attachments.

The Hoffman Mini Lengthener distractor system was used on all of the animals in this experiment (Howmedica, Rutherford NJ).

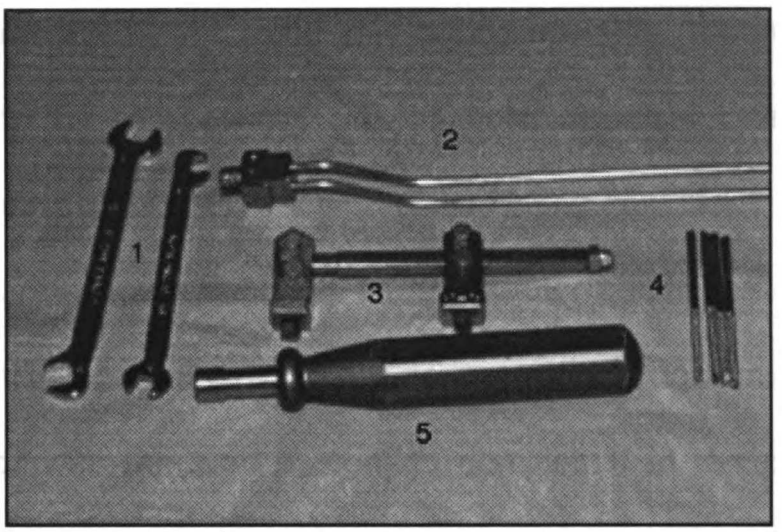


Figure 4: Distraction device components: (1) Wrenches for adjusting support orientation on the distractor; (2) pin paralleling guide; (3) Howmedica Hoffman Mini Lengthener unilateral extraoral distractor device (one support leg is on the left, one in the middle, and the activation nut at the end on the right side); (4) 50 mm x 1.5 mm diameter bone pins; (5) activation wrench.

Stab incisions to perforate the skin were made and two 1.5-mm diameter pin holes drilled into the lateral aspect of the mandibular ramus on both sides adjacent to the intended osteotomy site using a 1.5-mm diameter bone drill (Howmedica High Performance Gray, 1.5 mm x 50 mm; Howmedica, Rutherford NJ). The paralleling guide was used to correctly orient the pin holes. Four 50 mm x 1.5 mm diameter bone pins were manually screwed into the holes and bicortical threading visually confirmed. The unilateral distractor device was then placed

passively on the support pins and lightly secured to stabilize the mandible during the osteotomy. The device was oriented such that the hexagonal activation nut portion was facing upwards (towards the animal's ears) for easy access. A horizontal bicortical osteotomy was performed using a 0.4 x 26 x 4 mm reciprocating saw blade (SIM Medical 440/726, South Bend IN) on an air-rotor handpiece (Aesculap ELAN-E, Tuttlingen Germany) under normal saline irrigation, after which the distractor was removed to confirm completion of the osteotomy. Any remaining attached cortical bone was split using a mallet and chisel. Once a complete corticotomy was confirmed, the distractor was tightly secured, making sure the two mandibular segments were flush and not torqued (through visual inspection), and the support legs adjusted so the vector of distraction was as parallel to the ramus as possible. Along the posterior edge of the ramus on both sides of the osteotomy, a 1 mm diameter hole was drilled and a 3 mm length of 0.028" diameter round stainless steel wire (Ormco, Glendora CA) was tapped into each hole to mark the bone. The periosteum was sutured with 4-0 Vicryl (Ethicon, Somerville NJ), after which, a 2 mm x 1.5 mm diameter hole was drilled perpendicular to the lateral face of the ramus on both sides adjacent to the osteotomy. A metal-gelatin matrix was

injected into each of these holes up to the level of the periosteum to mark the periosteum. Closure was done in layers with 4-0 Vicryl suture. The skin was closed using 4-0 nylon suture. The bone pins were cut flush using orthopedic pin cutters and the wound site cleaned with Nolvasan chlorhexidine solution (Ft. Dodge Animal Health, Fort Dodge IA). 2% mupirocin antibiotic ointment (Bactoderm; Pfizer, New York NY) was also applied to reduce the risk of infection. Any sharp pin edges were covered either with segments of 19 x 7/8 plastic butterfly-needle tubing material (Abbott Hospitals, North Chicago IL) or surgical tape. Buprenorphine (0.01 mg/kg IM, Reckitt & Colman, Richmond VA) was administered for pain control immediately following surgery and q8h as needed. Surgerized animals were housed in cages lined with fine-mesh screen material or plexiglass sheets (1/8" thick, perforated at the corners for wire fixation to the cage walls) to prevent the device from being caught on the cage and becoming dislodged. Paired animals were separated following surgery so they could not bite at the each other's distractors during play. Chlorhexidine solution and mupirocin was applied daily to keep the pin-sites clean. Animals were fed *ad libitum* on a soft food diet (moistened pig chow). The skin sutures were removed 10 days post-surgically.

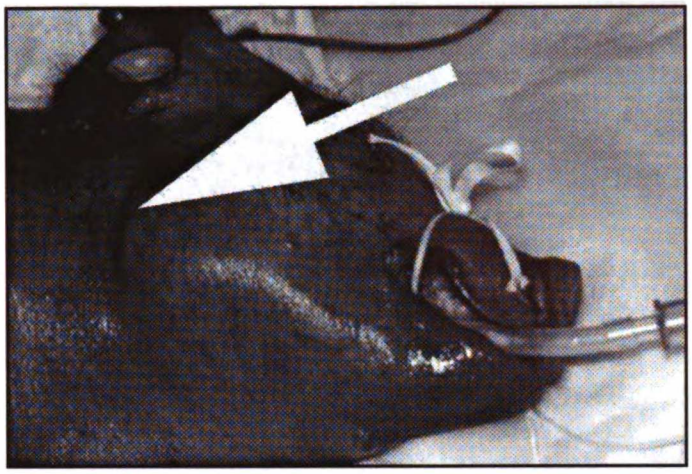


Figure 5: Area where the incision is made (arrow).

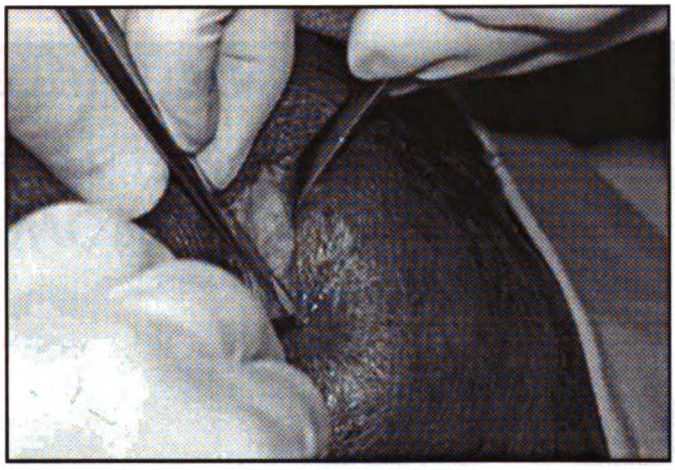


Figure 6: Initial incision and dissection of masseter.

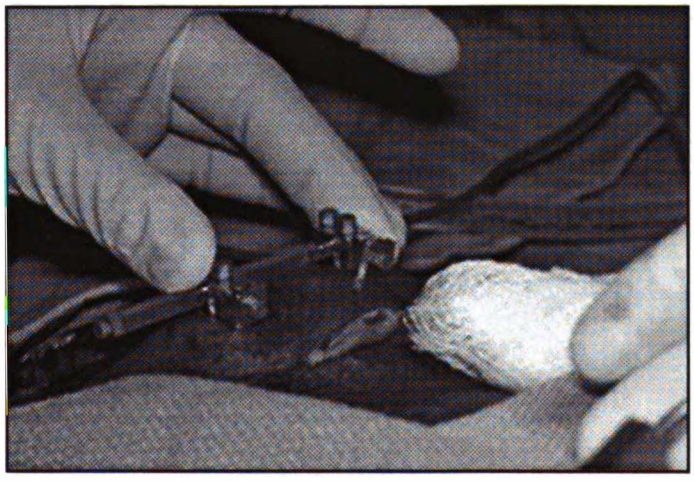


Figure 7: Pin site determination. Location is based on approximate location of the distractor device.

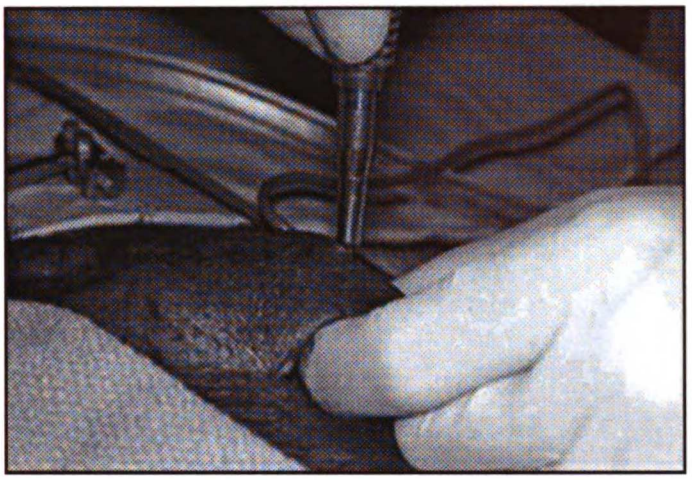


Figure 8: Pin hole placement. Subsequent pin holes were positioned using the pin paralleling guide.

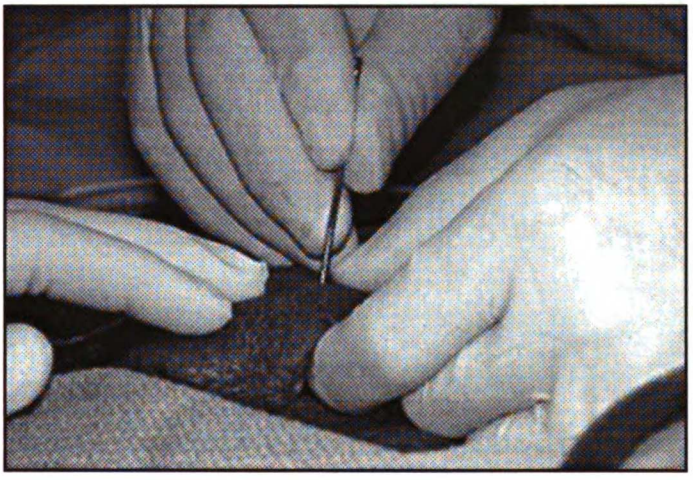


Figure 9: Pin placement. Hole is manually located.

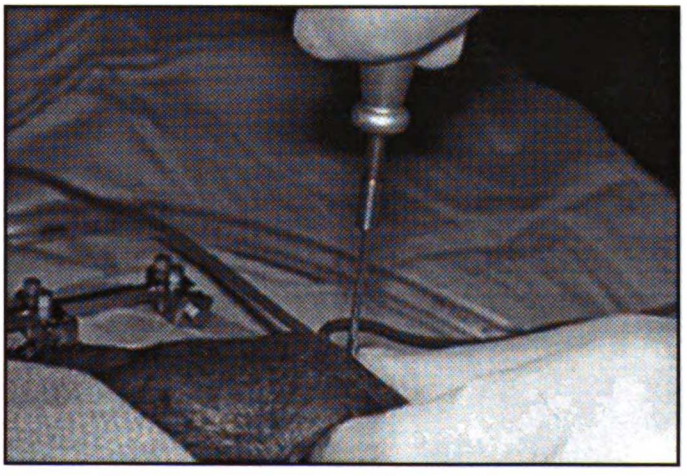


Figure 10: Pin tightening with pin wrench (not shown in Figure 4).

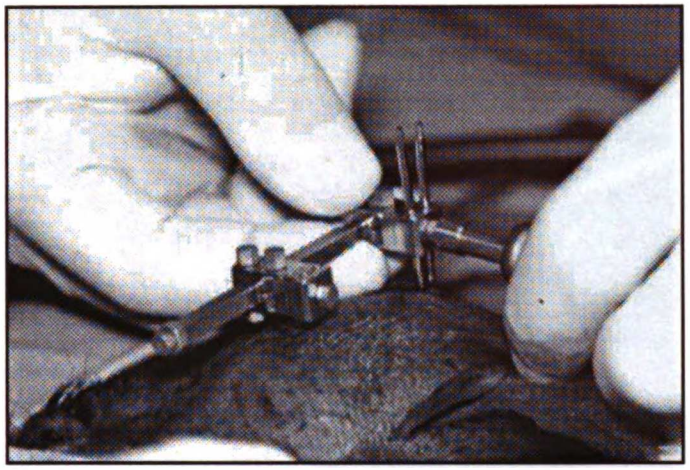


Figure 11: Preliminary device placement. Distractor support members are adjusted to passively orient around pins.

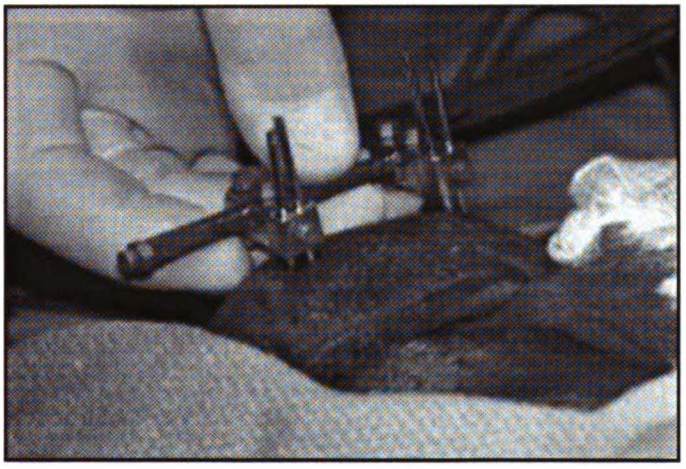


Figure 12: Secured device. The distractor holds the bony segments together during the osteotomy.

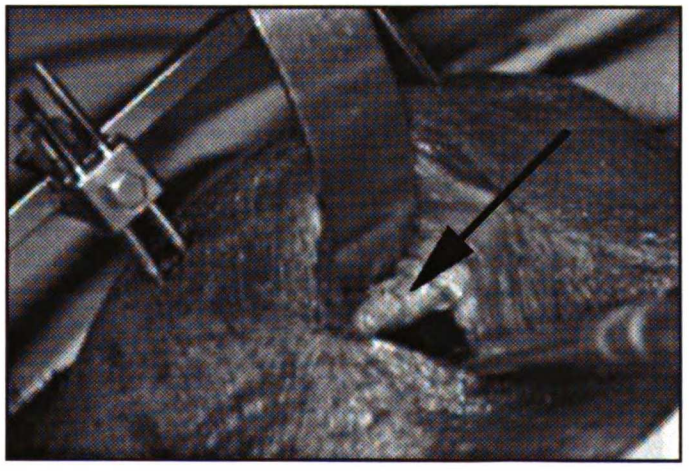


Figure 13: Osteotomy (arrow).

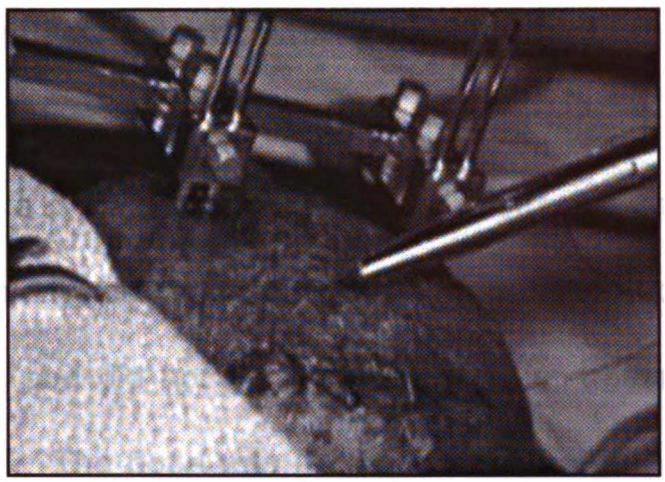


Figure 14: Suturing. Skin is closed using 4-0 nylon after the periosteum is sutured and the metal markers placed.

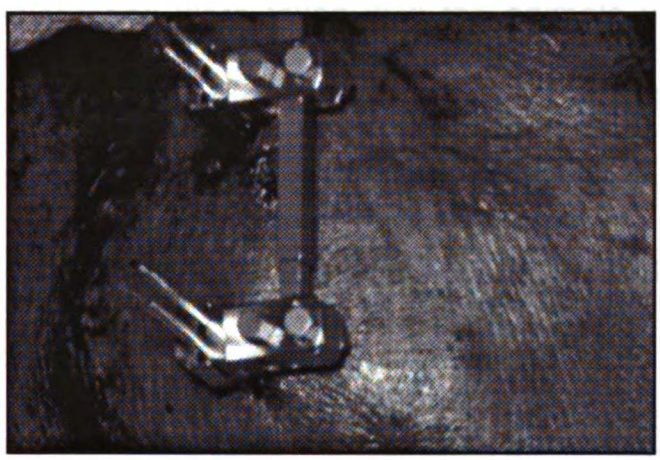


Figure 15: Device placement complete. Pins will be cut flush against the support legs.

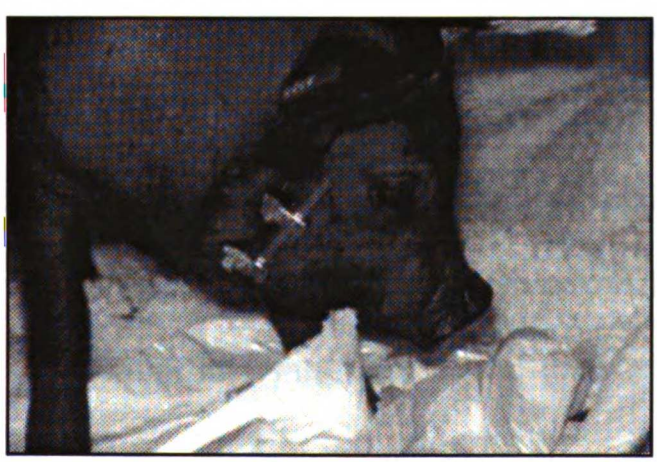


Figure 16: Rapid post-surgical recovery.

D.3 Device Activation and Radiographs

Following a 3 day latency period, the distractor was activated at a rate of 1 mm/day for fifteen days. Animals tolerated the activations well, and did not require anesthesia or treats in order to allow activation. The bone was allowed to mature for 15 days after the last activation before biopsies were taken (consolidation period).

Weekly radiographs were taken with the animals under light inhalation anesthetic (isoflurane, 1 to 4 percent). No other medication was required. Radiographs were taken using a table-mounted radiograph unit (Minxray HF80, Northbrook IL) at a fixed distance of 32" from the focal point. The animals' weight and head dimensions (height and width) were measured to determine the exposure time/kVp (0.18-0.40s, 65-70 kVp). Dorsoventral and lateral cephalograms were taken on Kodak T-Mat TML/RA-1 24 x 30 cm film in Kodak X-Omatic Lanex fine screen cassettes (Eastman Kodak, Rochester NY). Animals were oriented towards the x-ray beam using padded cushions around the facial region for support. Radiographs were taken immediately after surgery, at one-week intervals, and at the time of biopsy (6

total). The surgical site was cleaned with chlorhexidine and mupirocin applied at each radiographic event.

D.4 Biopsies

At the time of biopsy, the animals were sedated with ketamine HCl (20 mg/kg IM), xylazine (2 mg/kg IM), and atropine (0.04 mg/kg IM). The masseter muscles on both sides were carefully exposed and 4-0 silk-suture knots and ties placed at measured distances in the anterior and posterior superficial regions of the muscle.

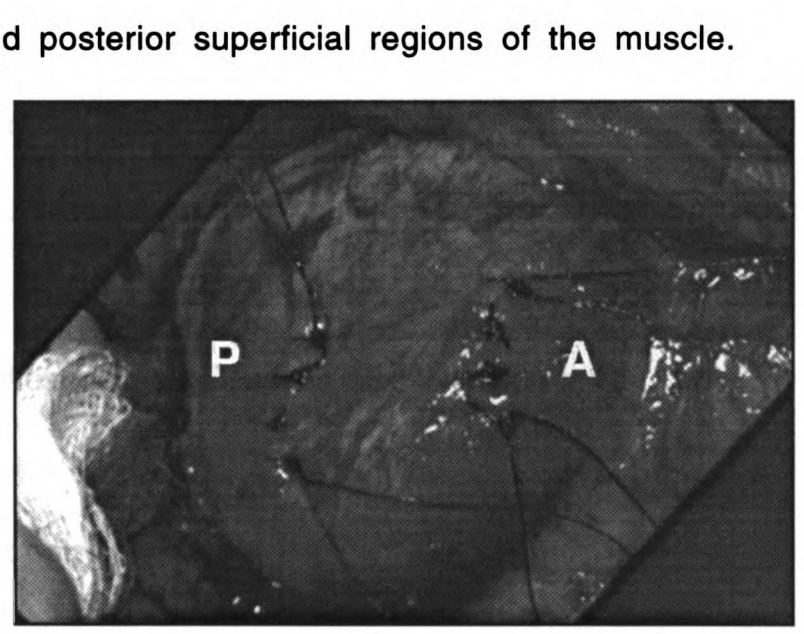


Figure 17: Right (control) masseter of Pig C exposed and labeled for biopsy (P = posterior, A = anterior). The outer ties (longer sutures) serve as handles for stretching the muscle to the pre-dissection length (distance between the inner knots) before the sample is snap frozen.

The entire muscle was then dissected and weighed, and the animal euthanized with sodium pentobarbital (150 mg/kg IV; Schering-Plough Animal Health, Kenilworth, NJ), followed by bilateral thoracotomy. 0.5 cm x 0.5 cm tissue blocks were dissected from the anterior, posterior, superficial, and deep regions of the muscle. Parallel samples (for sarcomere length studies at a future date) and transverse samples were obtained at each site, for a total of 8 samples per side. Each sample was stretched to its pre-dissection length before being snap-frozen in Tissue-Tek O.C.T. embedding solution (VWR Scientific, San Francisco CA) at -70°C with dry ice/isopentane. The frozen sections were coded and stored at -80°C. The pig skulls were then dissected, stripped of the soft tissues, and sent for dermestid-beetle processing to remove residual soft tissue (California Academy of Science, San Francisco CA).

D.5 Histochemical staining

Serial transverse sections, 5 μm thick were cut in a cryostat (Carl Zeiss HM505, Thornwood NY) at -25° C onto glass slides (Fisher Superfrost plus; Fisher Scientific, Pittsburgh, PA). H/E staining was

performed on each block to confirm the quality of each section series. Myofibrillar ATPase activity was determined with ATP-ase staining at pH 9.4 after alkaline (pH 10.3) and acid (pH 4.3 and 4.6) preincubation.[19,60] Acidic preincubation was carried out for 20 minutes in HCl-adjusted 0.1M Michaelis sodium barbiturate-acetate buffer solution (B6632; Sigma, St. Louis MO). Alkaline preincubation was done in 25 mM sodium barbiturate and 45 mM $CaCl_2$ adjusted with 0.1 M NaOH. All incubations were carried out at 21°C. Following preincubation, the sections were immersed for 20 minutes at room temperature in a freshly-prepared solution of 2.0 mL 0.1 M barbital buffer, 1.0 mL 0.17 M $CaCl_2$, 7.0 mL DI H_2O, and 25 mg ATP (Sigma), at pH 9.4 (9.39-9.41). Sections were washed 3x with 1% $CaCl_2$ in 10 minutes, washed 1x in 2% $CoCl_2O$ for 3 minutes, washed 1x in DI H_2O for 60 seconds, fixed in 1% ammonium sulfide (Aldrich, Milwaukee WI) in DI H_2O under the hood for exactly 40 seconds, and washed in tap water 3-4x. Sections were sequentially dehydrated in ethanol/xylene (95%, 100%, 100% EtOH, 100%, 100% xylene) and mounted with Permount (Fisher). These reactions were carried out in disposable plastic slide-mailers (Evergreen Scientific, Vernon CA) due to the volatility of the ammonium sulfide.

For capillary staining, sections were immersed in 1% paraformaldehyde in TBS with 1 mM $CaCl_2$ for 5 minutes, and thereafter rinsed 3 x 5 minutes in TBS. Sections were then fixed in 0.5% periodic acid (Aldrich) for 5 minutes and rinsed 3 x 5 minutes in TBS. Endogenous biotin was blocked by incubation with ExtrAvidin (Sigma) 1:100 in washing buffer (TBS containing 1 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$) for 30 minutes and rinsed 3 x 5 minutes in washing buffer. Sections were incubated overnight at 4° C with LTA (*Lotus tetragonolobus*, winged or asparagus pea; Sigma) diluted to a concentration of 10 µg/mL in washing buffer, rinsed 3x5 minutes in TBS, incubated for 30 minutes at room temperature with alkaline-phosphatase-conjugated avidin (diluted 1:300 with TBS pH 7.6, 0.1M, Sigma), washed in TBS (pH 7.2, 0.1 M) 3 x 5 minutes, and visualized through incubation for 20-30 minutes at room temperature in a medium containing 10 mL Tris buffer (pH 9.5, 0.1 M), 10 mM $MgCl_2$, 2.5 mg 5-bromo-4-chloro-indoxyl phosphate (Aldrich) in 5 mL DMF, 3 mg NBT (Aldrich), and 2 mg levamisole (Sigma).

D.6 Histochemical analysis

Images of stained sections were digitally captured using a CCD video system (Olympus BH microscope, Japan; JVC TK-870U color video camera head, Japan; RasterOps MediaGrabber v2.1 video capture software, Santa Clara CA; Apple Macintosh OS 7.5.5, Cupertino CA). Equivalent fields from the different ATPase reactions were identified and captured at 200x magnification and the fiber type determined from the myosin-ATPase reactions using the classification developed by Brooke and Kaiser.[4,5] Lightly stained fibers following alkaline preincubation (pH 10.3) were classified as type I fibers, moderately stained fibers labeled intermediate (IM), and darkly stained fibers called type II. Type II fibers which showed an inhibition reaction (light stain) at both pH 4.3 and 4.6 were classified as type IIA, and fibers which showed an inhibition reaction only after pH 4.3 were labeled type IIB. Fibers which showed dark staining at both pH 4.3 and 4.6 were labeled type IIC.

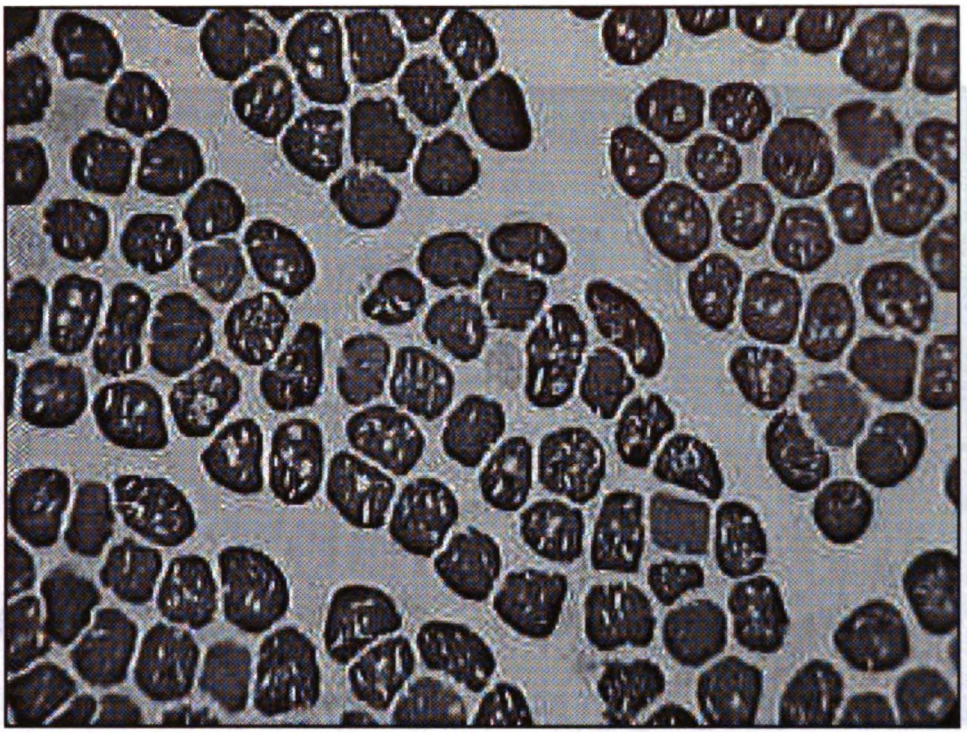


Figure 18: Myosin-ATPase staining at pH 4.6 (Superficial posterior control fibers of Pig B). x200.

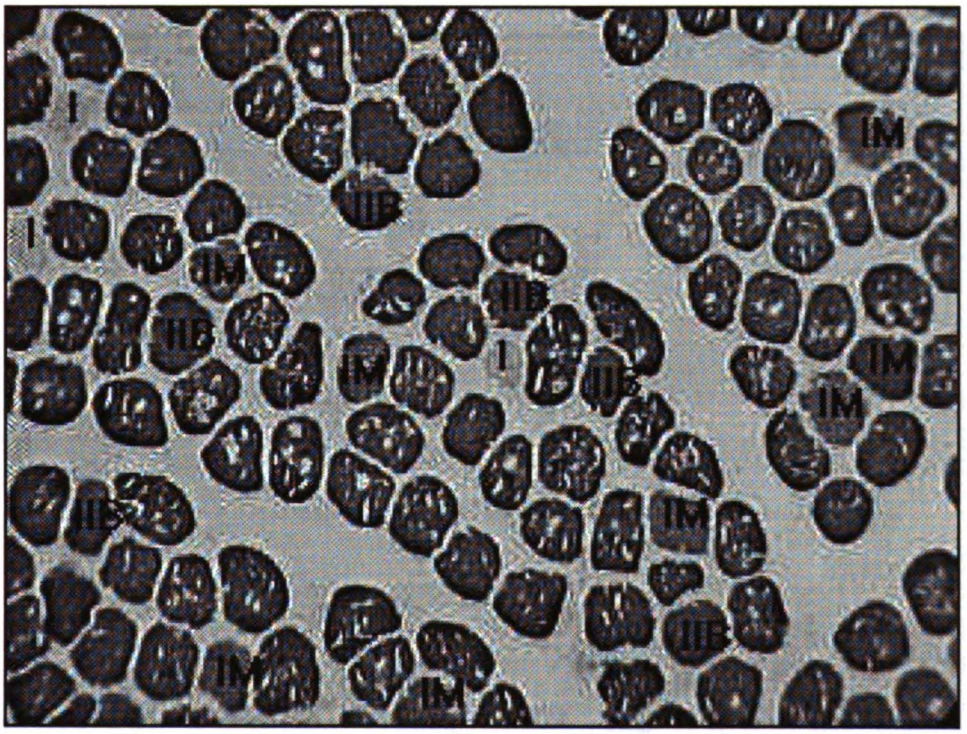


Figure 19: Examples of identified fiber types.

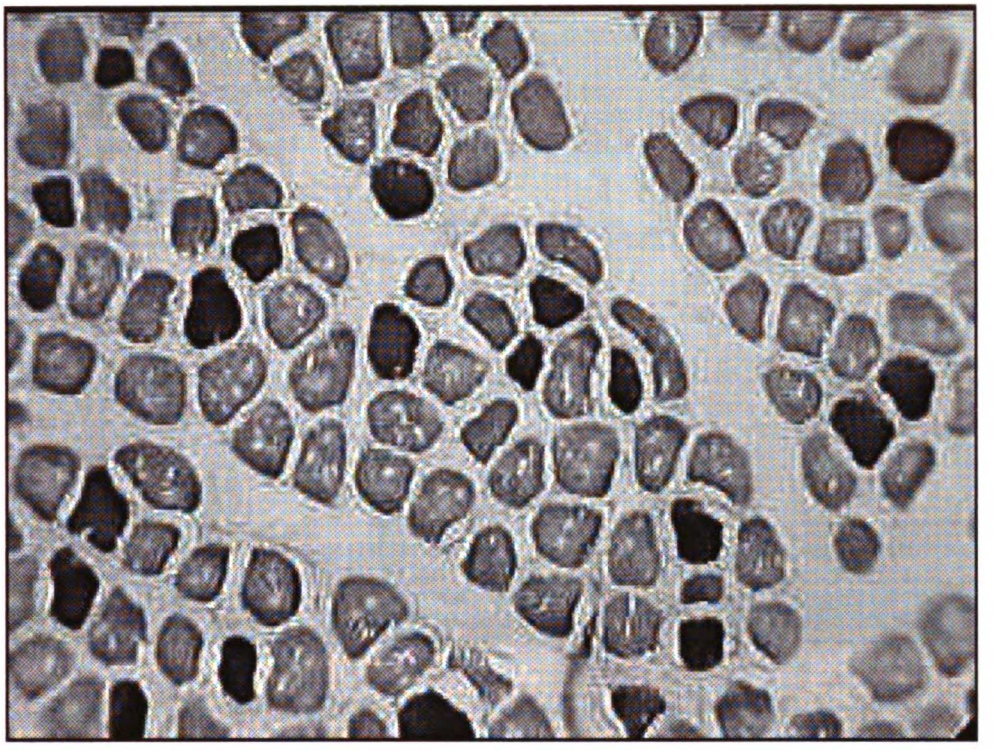


Figure 20: Myosin-ATPase staining at pH 10.6. x200.

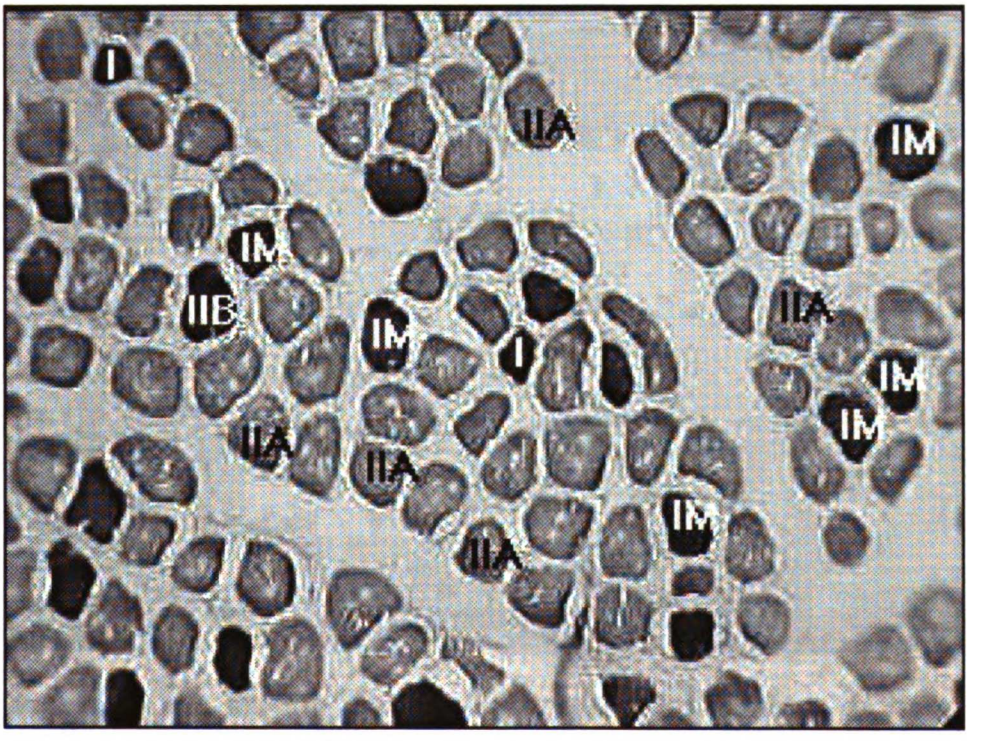


Figure 21: Examples of typed fibers.

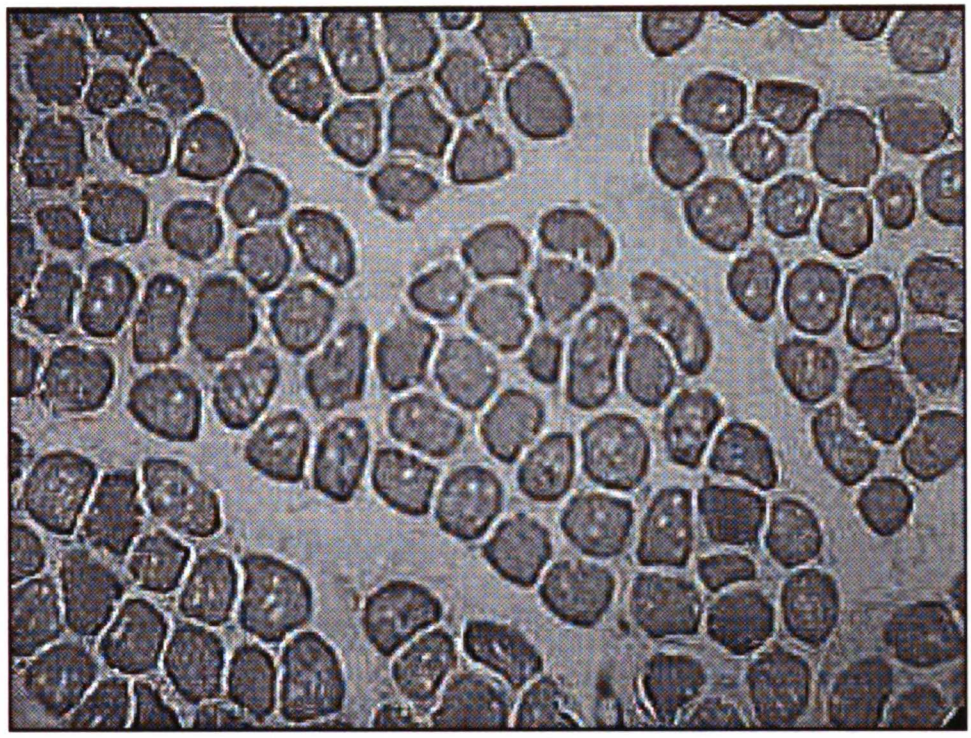


Figure 22: Myosin-ATPase staining at pH 4.3. No type IIC fibers are present. x200.

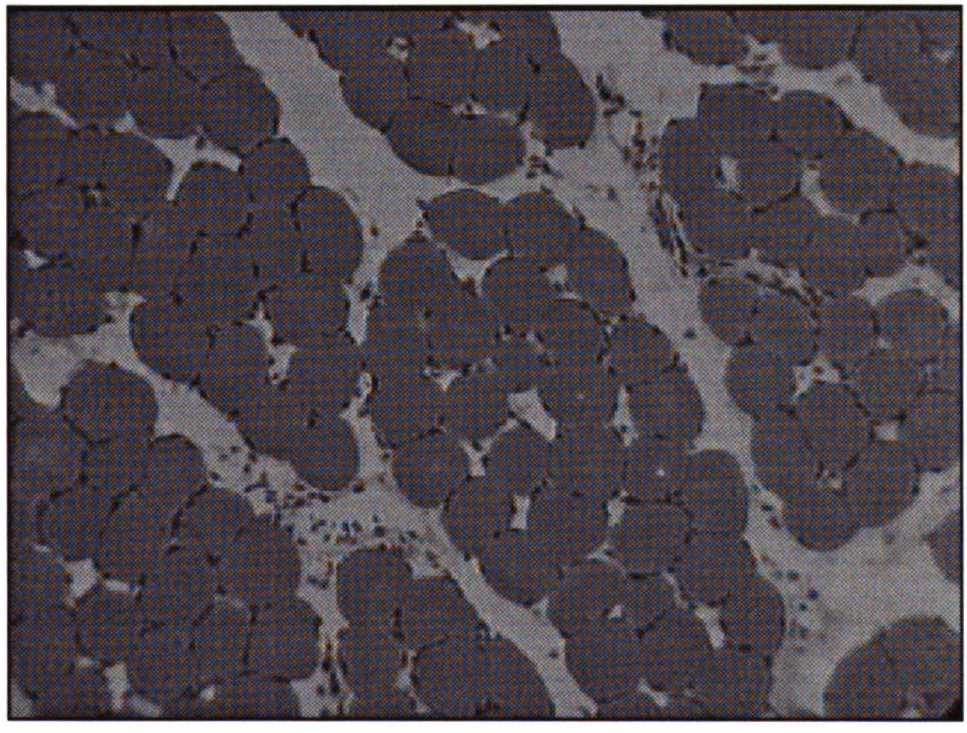


Figure 23: H/E staining. Fibers are matched to same fields as ATP-ase sections in Figures 18-22. x200.

The average fiber cross-sectional area was determined from the H/E slide sections using public-domain program NIH Image v1.61 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). All sections were coded with a random numerical assignment from a master list and the examiner blinded to the identity of each section until the totals were compiled to minimize bias during fiber counting and image processing. Artifacts and partial fibers from the captured fields were digitally removed using Adobe Photoshop (v 4.0; Adobe Systems, Mountain View CA).

The total number of pixels at maximum threshold was divided by the number of fibers in the field to give an average pixel count per fiber, which represented the mean fiber area. The experimental side was divided by the control side to give a cross-sectional area ratio (E/C).

To calculate the number of fibers per unit area, the fascicle area was computed using NIH Image and normalized to an arbitrary unit area. The number of fibers enclosed by the fascicle(s) was then scaled by the normalizing factor. The scaled number on the experimental side was divided by the corresponding number of fibers

on the control side (also normalized) to yield a ratio indicating the overall percent change in fiber number. Anterior sites were compared to posterior sites, and superficial sites compared to deep sites for both fiber count and fiber area. Capillary density was determined by dividing the number of peripheral capillaries by the number of fibers enclosed.

E. Results

E.1 Clinical Evaluation

E.1.1 Animal A:

Pig A was surgerized without complication at the age of 8 weeks on the right side. Only one animal was surgerized. Recovery was extremely rapid, and the animal was ambulatory with an excellent appetite approximately 20 minutes after recovery from anesthesia. 18 mm distraction was achieved, after which, the animal inadvertently caught the distractor in the grill aperture of the cage and pulled it completely off (the goal was 21 mm for the first animal—later scaled down to 15 mm to reduce the experimental cycle time). The pin holes had been completely stripped, but the osteotomy site was stable, so

the animal was put up for adoption without further action. The device had been attached for 21 days before the failure occurred.

Activations were made using softened pig chow as a diversion, which worked quite well initially. As the animal grew older, however, this associative conditioning caused the animal to become easily excitable, making activation extremely difficult.

Periosteal markers consisting of spherical titanium alloy (average diameter = 100 microns; Nuclear Metals, Concord MA) suspended in 10% gelatin (w/v, Knox unflavored; Nabisco, East Hanover NJ) were embedded along with the steel bone markers. Lacking radiodensity, the titanium markers were difficult to visualize radiographically (see Figure 27).

E.1.2 Animal B:

Pig B was surgerized without complication on the left side at the age of 5 weeks. Post-operative recovery time was significantly shortened by keeping the animal in the company of her sibling, Pig C. The cage was completely lined with a fine-mesh window-screen material to prevent the device from getting caught. Device activation was accomplished in the absence of food, substituting toys and play as a

diversion. This worked just as well as food and did not lead to behavior-management problems. The spherical-titanium matrix was replaced with a spherical-silver matrix (Tytin spherical alloy, average diameter = 10 microns; SDS-Kerr, Orange CA) to increase the implant radiodensity.

The two animals were separated 2 weeks post-operatively because they were observed biting at each others' distractors. Partial device loosening was noted on post-surgery day 16. The cause was believed to be device biting by Pig C. The distractor was completely dislodged by day 18 and had to be resecured using 2 mm diameter pins under light inhalation anesthesia. Activation was held at 15 mm for 4 days, after which, the device was found dislodged again (day 23 post-surgery). The device could not be reattached because the holes had been completely stripped, so the surgical site was allowed to heal for 15 days without the device in place. Biopsies were then taken and the animal euthanized.

Examination of the biopsied muscles revealed a scarred, irregular, and narrower masseter on the experimental side. Fibrous encapsulation was also noted around the silver-particle matrix residue. The control side was even-textured with no observable scarring.

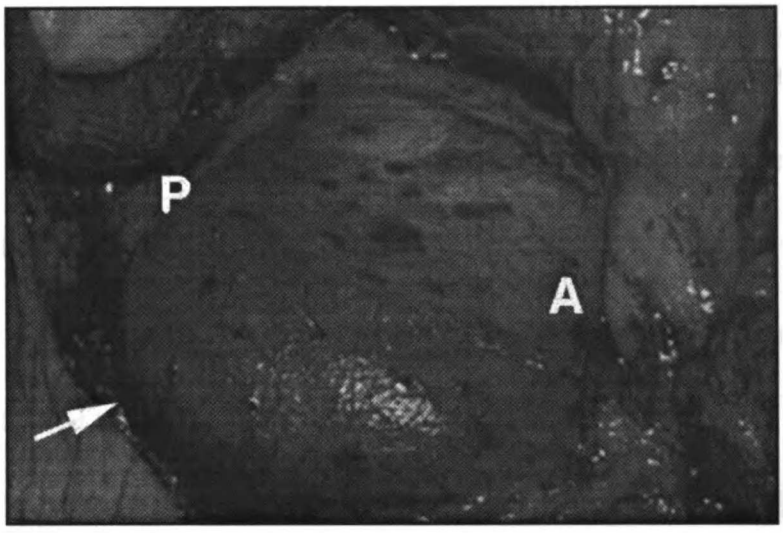


Figure 24: Pig B, right masseter (control side). P = posterior, A = anterior. The arrow points to the gonial angle of the mandible. Note the uniform surface texture.

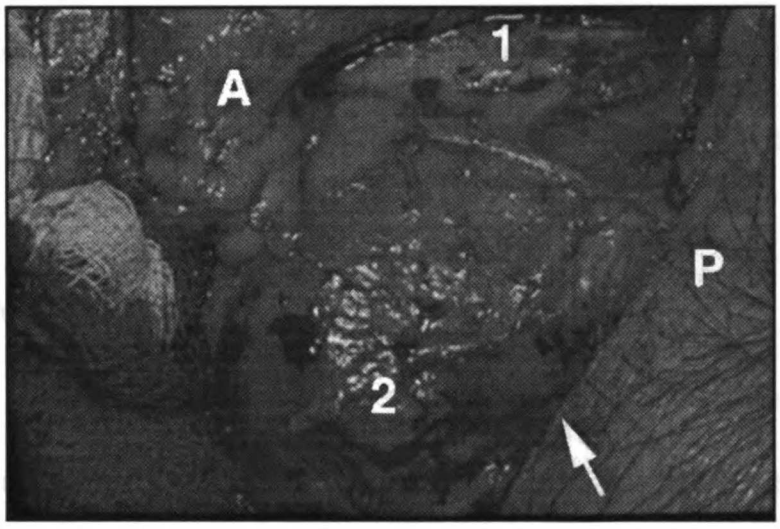


Figure 25: Pig B, left masseter (experimental side). A = anterior, P = posterior. The arrow points to the gonial angle of the mandible. Note the scarring at the pin sites (1,2), the irregular texture, as well as the decreased width relative to the control side.

E.1.3 Animal C:

Pig C was surgerized on the same day and in the same manner as Pig B, also on the left side. Surgery and post-operative recovery occurred without complications. The distractor device was found lying on the floor of the cage 11 days into the consolidation phase (device failure on day 29 post-surgery). The cause of the loss was unknown, but was later believed to be device leveraging complicated by pin-tract infection. The device was not reattached due to pin-hole stripping. The bone was allowed to consolidate for an additional 4 days before the muscles were biopsied and the animal euthanized. Clinical presentation of the biopsied muscles was similar to that observed in Pig B.

E.1.4 Animal D:

Pig D was surgerized without complication at the age of 6 weeks on the left side, with rapid and uneventful post-operative recovery. The stainless steel bone markers were continued, but the silver-matrix periosteal markers were discontinued because the material could not be injected without radio-obstructive dispersion (see Figure 28).

The post-operative protocol was modified to include systemic antibiotic administration (erythromycin suspension 15 mg/kg BID x

10d; Barr Labs, Pomona NY) and topical antibiotic ointment administration around the sutures and pin sites (2% mupirocin BID, SID Sundays and holidays) to reduce the risk of device loosening from pin-tract infection. Animal D was separated from its sibling (Pig E) immediately following post-surgical recovery to prevent device loosening from play.

On day 13 post-surgery, loosening of the upper pins was observed. Three days later, the device was found on the floor of the cage (device failure at day 15, 9 mm activation completed). The cause of device failure was unknown. A 3-inch hole was found chewed into the screen lining of the cage, but whether this contributed to device failure could not be determined. Reattachment was not attempted due to pin-hole stripping, and the bone was allowed to consolidate for 15 days before the muscles were biopsied and the animal euthanized.

E.1.5 Animal E:

Pig E was surgerized without complication at the age of 6 weeks on the left side, with rapid and uneventful post-operative recovery. The device came off after only 1 mm activation (day 5 post-surgery), but the upper bone pins were still attached to the animal.

Reattachment surgery was attempted, but the bony segment containing the upper pin holes had been fractured (probably during transport to surgery), so the animal had to be euthanized due to insufficient fixation required for jaw healing and function.

E.1.6 Animal F:

Pig G was sick and dehydrated upon arrival from the vendor. Rehabilitation was unsuccessful, and the animal had to be euthanized.

E.1.7 Animal G:

Pig was surgerized without complication on the right side at the age of 10 weeks, with rapid and uneventful post-operative recovery. After surgery, this animal was housed in a plexiglass-lined cage to eliminate any chance of device catch. During recovery from anesthesia, placement of an E-collar was attempted to prevent the animal from scratching at or rolling on the device. Midazolam (1 mg/kg IM; Roche Pharmaceuticals, Manati PR) was administered to sedate the animal. The E-collar had to be removed because insufficient time had been given for acclimation. As a substitute, gauze padding was layered around the device to help cushion any applied pressure.

Even with padding, the device still failed. It was found separated on day 6 post-surgery with the upper pins intact and the lower pins out. Reattachment surgery was performed, but the device came loose again 2 days later. A second reattachment surgery was performed and a circumferential head wrap used to help secure the device, but this did not work either (the device failed the next day). The bone was allowed to heal for 15 days, after which, the muscles were biopsied and the animal euthanized. Scar tissue was observed on the posterior region of the dissected masseter on the experimental side, but to a lesser degree than in any of the other animals.

E.2 Radiographic Evaluation

E.2.1 Animal A:

Based on the position of the stainless steel markers (uncorrected for magnification), approximately 13 mm vertical distraction was achieved. This distance remained the same throughout the consolidation period despite loss of the device. Unequal distraction appears to have taken place, with greater bony-plate separation in the posterior than in the anterior (approximately 2:1 ratio). Because the device faced anteriorly (see Figure 51), the implants and osteotomy

site were minimally obstructed. The titanium markers were difficult to visualize because of their low radiodensity. The bone pins had been inserted 5 mm beyond the medial cortical plate.

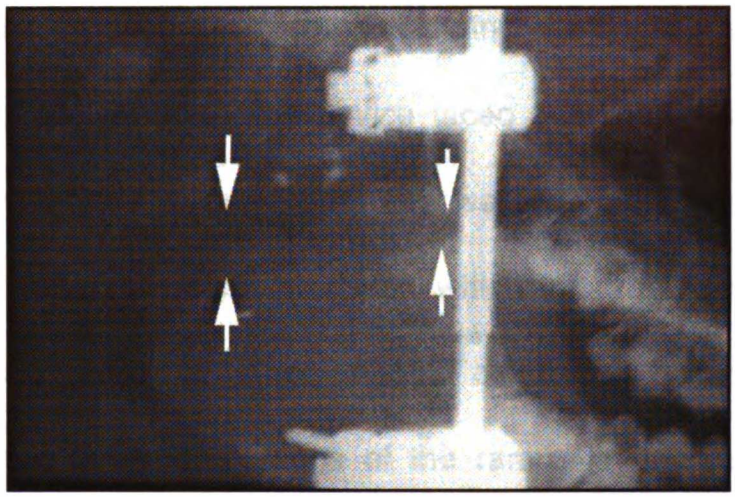


Figure 26: Unequal mandibular segment separation, Pig A. 65 kVp, 0.44s exposure time.

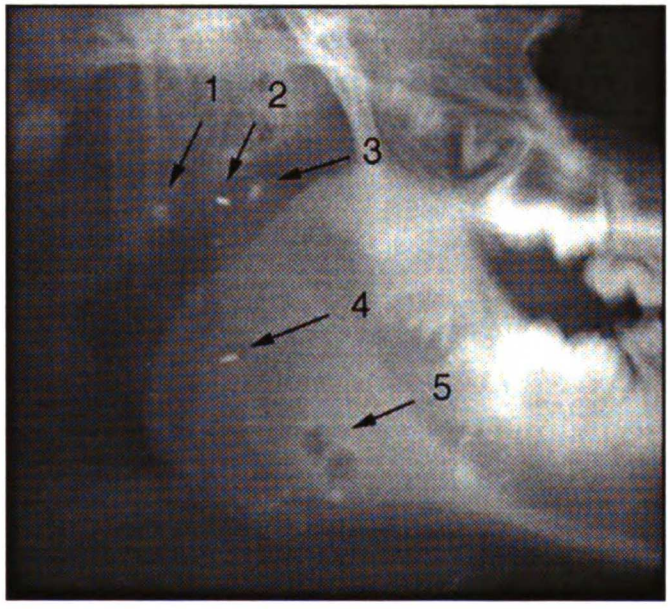


Figure 27: Pig A lateral cephalogram (post-device failure). Titanium matrix markers (1,3) were difficult to visualize due to their low radiodensity. Steel markers (2,4) were used to determine the amount of vertical distraction achieved. (5) Pin hole radiolucencies. 65 kVp, 0.44s exposure time.

E.2.2 Animal B:

Based on the position of the stainless steel markers (uncorrected for magnification), 5 mm vertical distraction was achieved. This distance did not shrink during the consolidation period despite loss of the device. The device faced posteriorly (see Figure 50) so the implants and osteotomy site were partially obstructed. Silver-matrix dispersion also interfered with radiographic visualization of the distraction site. Whether unequal distraction occurred between the anterior and posterior portions of the ramus is unclear. Radiolucencies from pin-tract infections were present around the pin sites. The bone pins had been inserted flush against the inner edge of the medial cortical plate, except for one upper pin which extended 12 mm beyond the medial cortical plate.

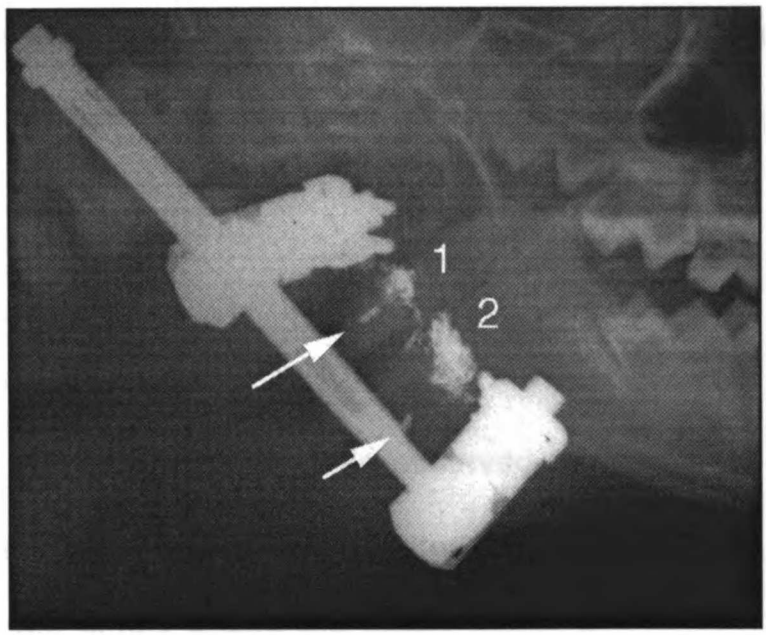


Figure 28: Lateral cephalogram of Pig B illustrating silver-matrix dispersion (1,2). Arrows point to steel implant markers. 70 kVp, 0.28s exposure time.

E.2.3 Animal C:

Based on the position of the stainless steel markers (uncorrected for magnification), 12 mm vertical distraction was achieved. This distance did not shrink during the consolidation period despite loss of the device. The device faced anteriorly so as to only minimally obstruct the view of the implants and osteotomy site; however, dispersion of the silver-matrix interfered with visualization of the distraction site. Whether unequal distraction occurred between the anterior and posterior ramus is unclear. Large radiolucencies from pin-tract infection were present around the pin sites. The bone pins

had been inserted flush against the inner edge of the medial cortical plate except for one upper pin which extended 7 mm beyond the medial cortical plate.

E.2.4 Animal D:

5 mm vertical distraction was observed based on the position of the stainless steel markers (uncorrected for magnification). This distance did not shrink during the consolidation period despite loss of the device. The device faced anteriorly so as to only minimally obstruct the view of the implants and osteotomy site. Unequal distraction appears to have taken place with greater bony-plate separation in the posterior than in the anterior (approximately 7:3 ratio). Two bone pins had been inserted flush against the inner edge of the medial cortical plate. Of the other two, one (upper) pin extended 4 mm beyond the medial cortical plate. The other (lower) pin extended 3 mm beyond the medial cortical plate.

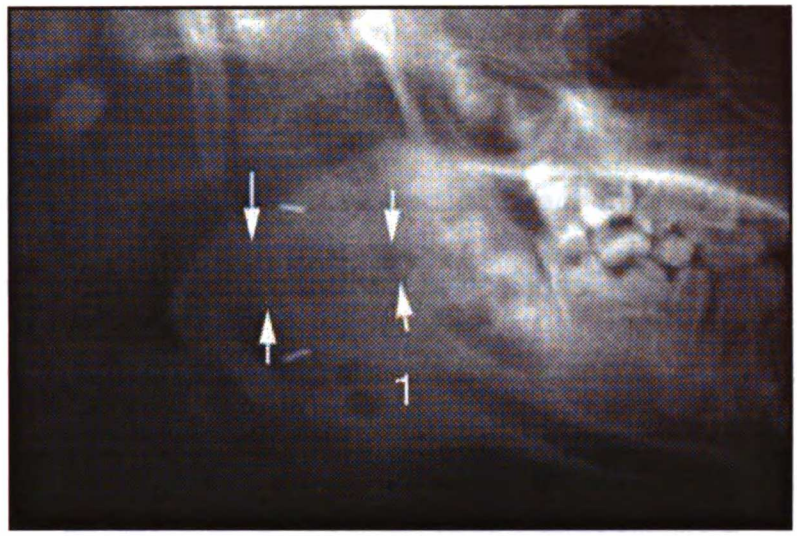


Figure 29: Disproportionate segment separation, Pig D. 70 kVp, 0.18 exposure time. Radiolucencies (1) are where the lower pins were placed.

E.2.5 Animal E:

The upper bone pins had been extended 2 mm beyond the inner edge of the medial cortical plate. The lower bone pins had been extended 3 mm beyond the inner edge of the medial cortical plate. The device faced posteriorly. Consequently, the steel markers were obstructed from view.

E.2.6 Animal G:

All four pins had been placed flush against the inner edge of the medial cortical plate. The device faced posteriorly. Consequently the steel markers were obstructed from view.

E.3 Skeletal Evaluation

Inspection of the skulls revealed pin-tract infections in animals B and C, which probably contributed to device loss. The holes in the mandible were significantly larger than the diameter of the pins.

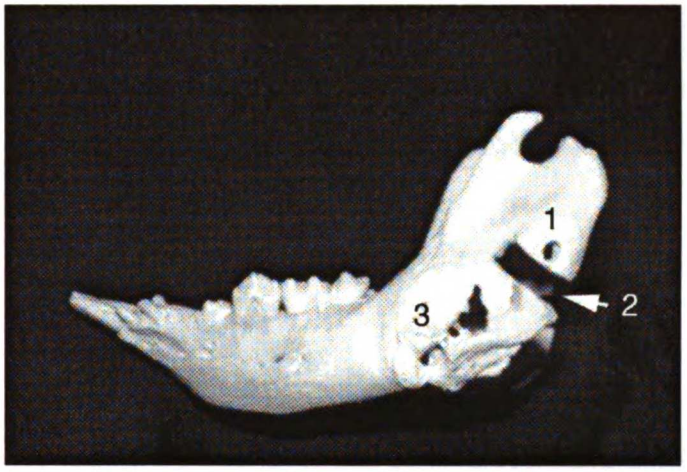


Figure 30: Pig B mandible. Holes (1,3) were caused by pin-tract infection. Notch (2) is from bone biopsy for future study.

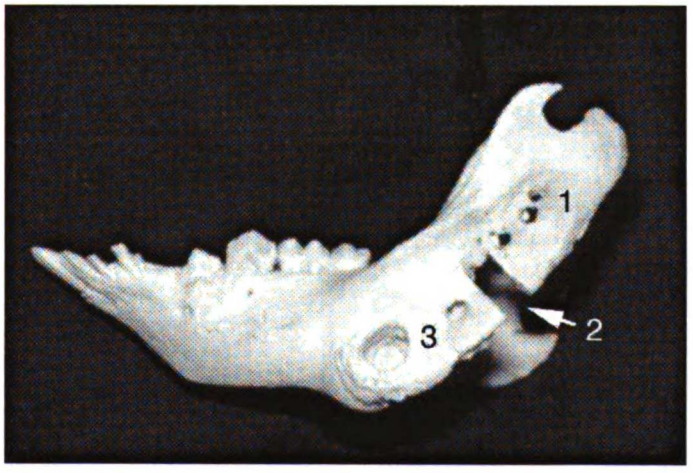


Figure 31: Pig C mandible. Holes (3) were caused by pin-tract infection. Upper pin holes (1) appear minimally affected by infection. Notch (2) is from bone biopsy for future study.

Osseus union was achieved in all distracted areas despite device failure. Distinctly noticeable was the formation of irregular bony exostoses around the osteotomy and pin sites. The ramus appeared to be thicker medio-laterally on 3 of the 4 skulls (B,C,G).

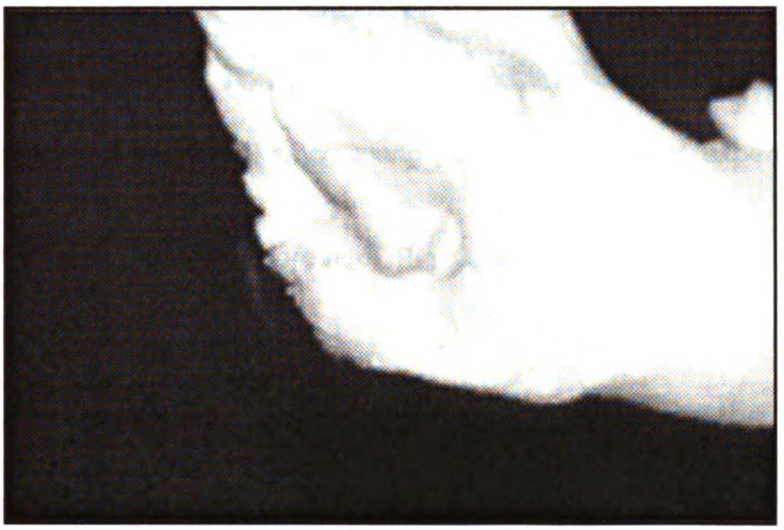


Figure 32: Irregular bone formation around osteotomy site of Pig G.

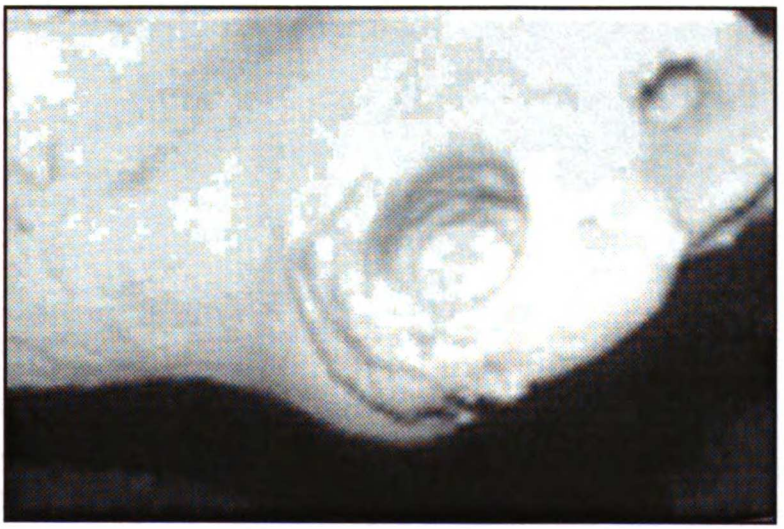
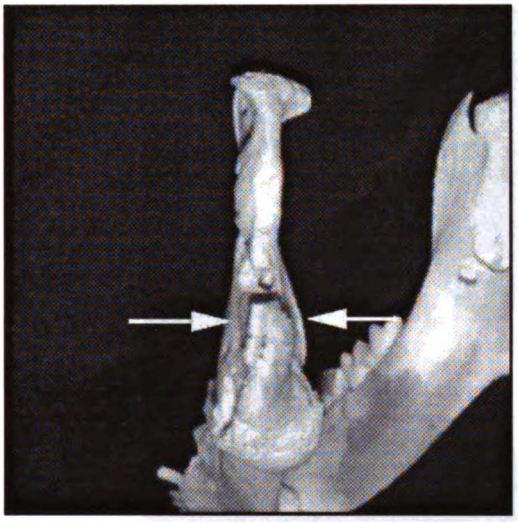


Figure 33: Exostosis formation around lower pin site of Pig C.



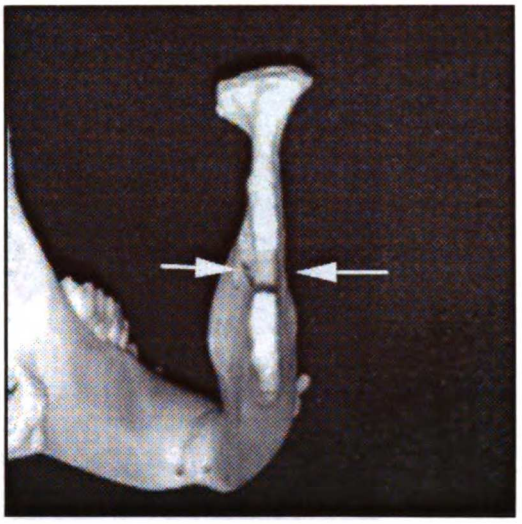


Figure 34 (left): Ramus thickening along posterior border of experimental side, Pig C.

Figure 35 (right): Posterior border of Pig C, control side.

The surgerized ramus was significantly more narrow in the antero-posterior dimension by an average of 5.9 mm or 17% (SD 7%, P<.02)

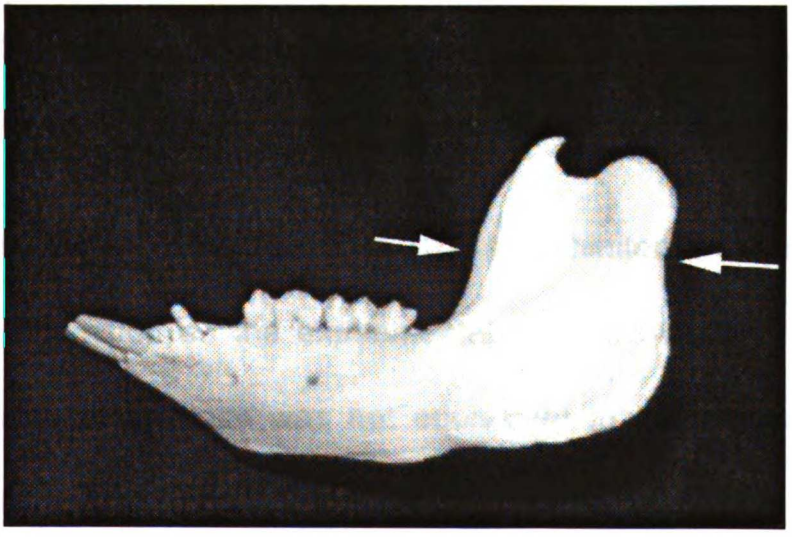


Figure 36: Ramus width of Pig G, control side.

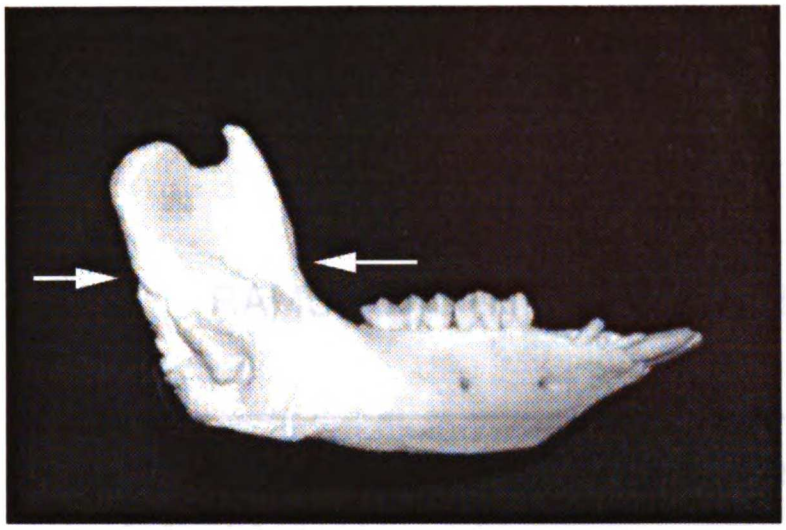


Figure 37: Ramus width of Pig G, experimental side. Decreased width is especially noticeable near the gonial angle.

ANIMAL	CONTROL (mm)	EXPERIMENTAL (mm)	DIFFERENCE (mm)	%
B	33.5	31.2	2.3	7%
C	34.2	26.7	7.5	22%
D	34.3	26.5	7.8	23%
G	37.3	31.5	5.8	16%
MEAN	34.8	29.0	5.9	17%
STD DEV	1.7	2.7	2.5	7%
P <	0.02			

Table 2: Ramus width differences between control and experimental sides.

The distracted mandibles (A,B,C,D) all exhibited an increase in the vertical dimension on the experimental side. However, the actual amount of vertical increase was not equivalent to the amount of vertical activation achieved.

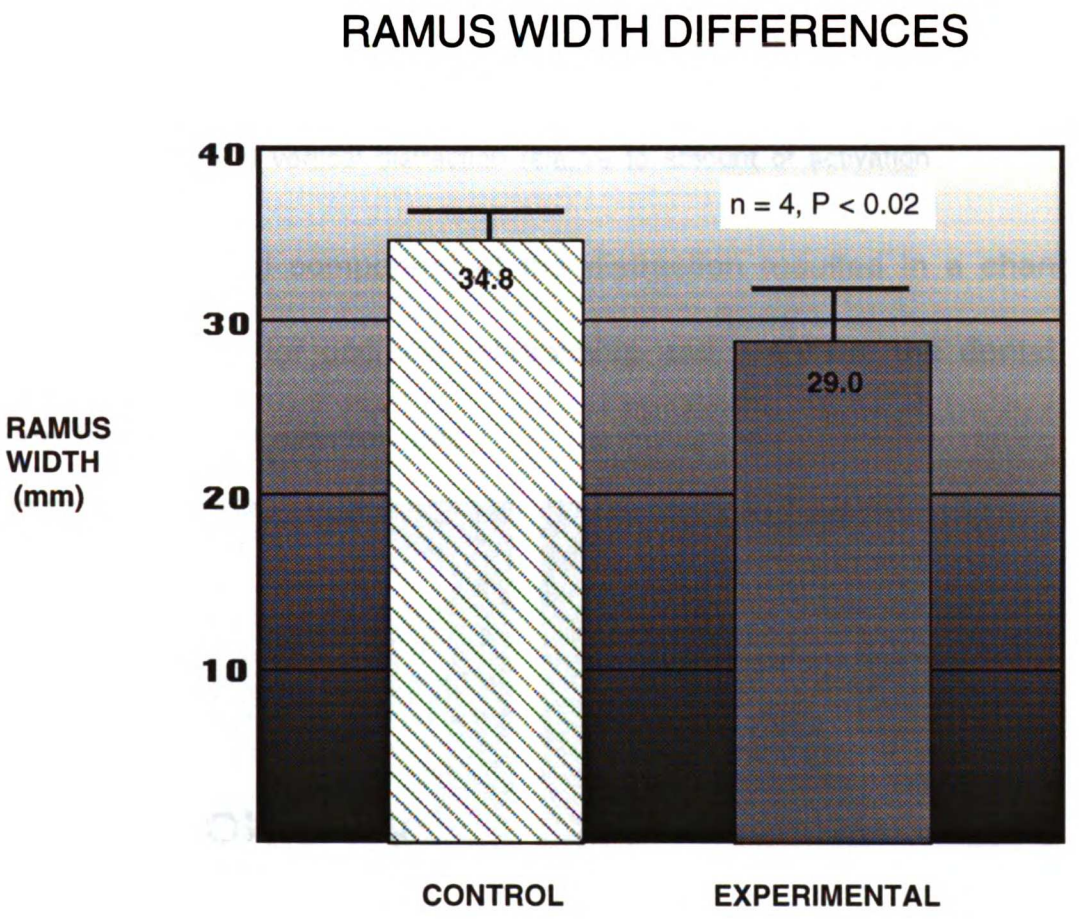


Figure 38: Graph summarizing width comparison.

ANIMAL	TOTAL ACTIVATION (mm)	VERTICAL CHANGE (mm)	%
A	18	13	72%
B	15	5	33%
C	15	12	80%
D	9	5	56%
		MEAN	60%
		STD DEV	21%

Table 3: Percentage of vertical distraction relative to amount of activation.

The horizontal component of the distraction resulted in a change in the antero-posterior occlusal relationship and a shift in the dental midline towards the control side.

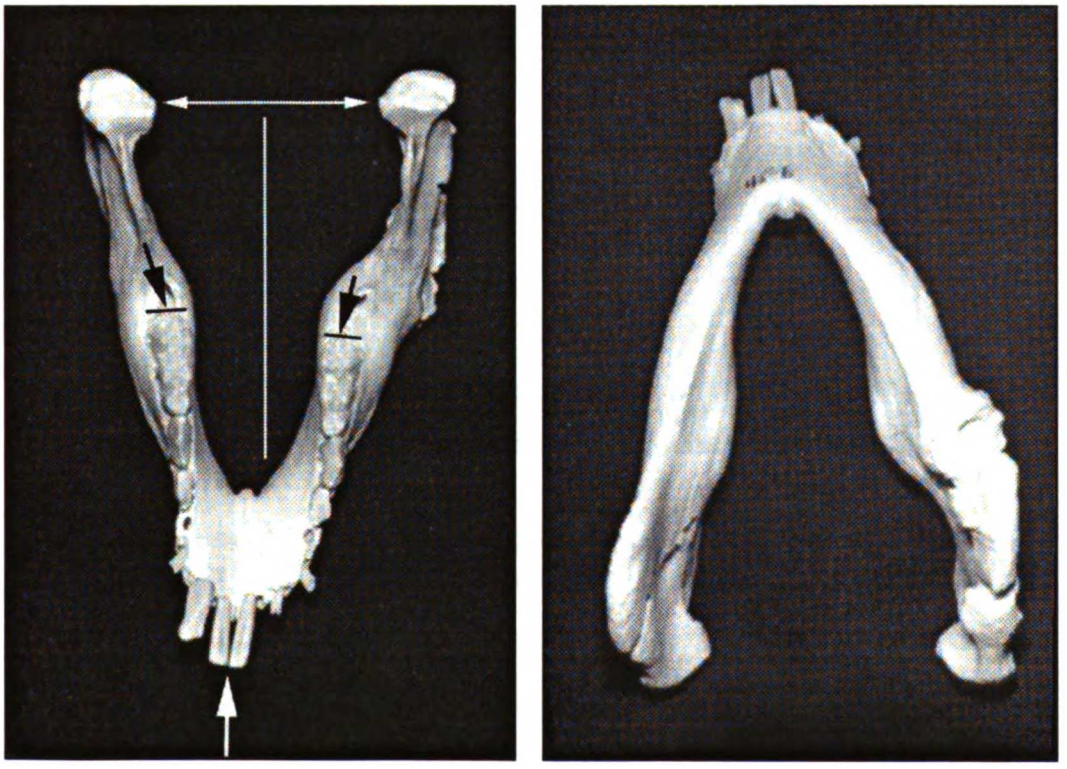


Figure 39 (left): Mandible, superior view (Pig B). Experimental side is on the animal's left. Note the dental asymmetry and midline deviation.

Figure 40 (right): Mandible, inferior view (Pig B). Note the bony exostosis formation on the experimental side.

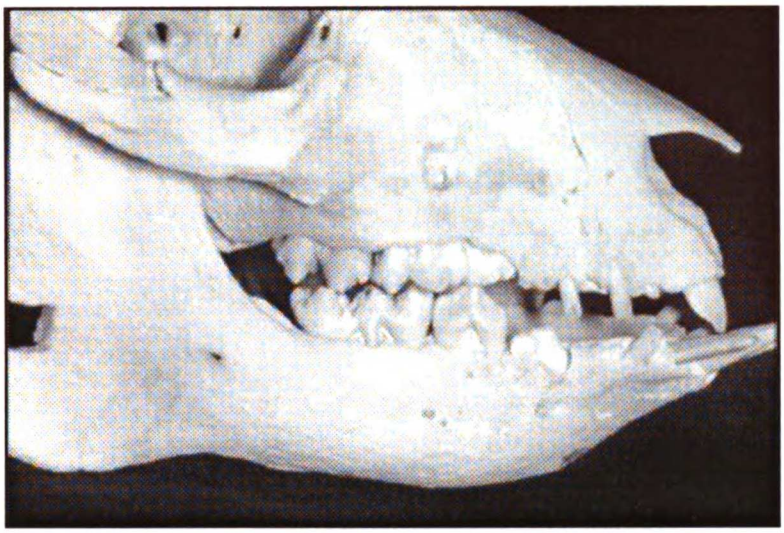


Figure 41: Buccal dentition, control side (Pig B).

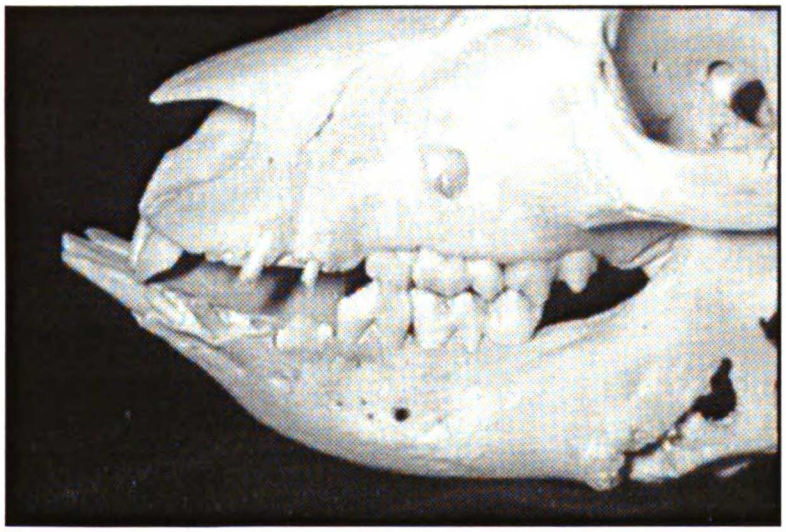


Figure 42: Buccal dentition, experimental side (Pig B). The lower molars are advanced more forward than the molars on the control side.

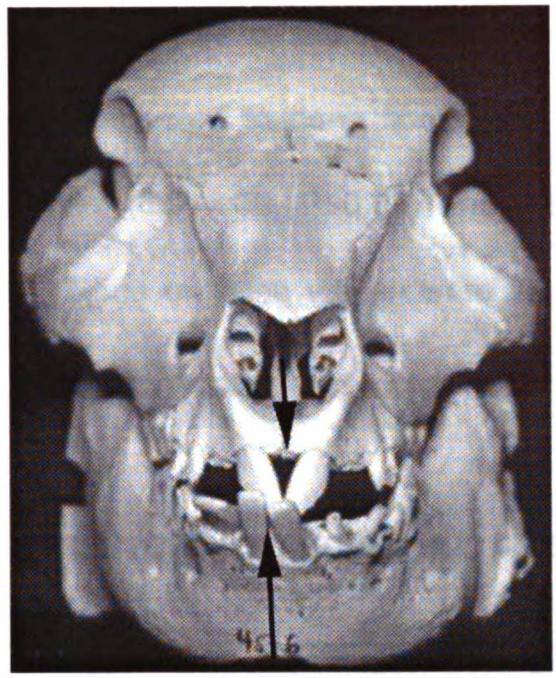


Figure 43: Skull, anterior view (Pig B). Note the midline deviation.

E.4 Histological Evaluation

E.4.1 Fiber Area

A total of 2,297 experimental fibers and 2,145 contralateral control fibers were measured for cross-sectional area. The average experimental/control ratio (E/C) was 0.89 (SD = 0.06, $P>0.05$). When anterior vs. posterior subgroups were compared, the anterior E/C ratio was 1.03 (SD = 0.01, $P>0.05$) and the posterior E/C ratio was 0.75 (SD = 0.11, $P>0.05$), indicating a mild increase in the average cross-sectional area of the anterior fibers and a moderate decrease in

the average cross-sectional area of the posterior fibers. When superficial fibers were compared to deep fibers, the E/C ratio was 0.96 for superficial fibers (SD =.05, P>0.05) and 0.83 for deep fibers (SD = 0.07, P>0.05). None of these values were significant due to insufficient sample size (n=2).

Table 4: MUSCLE FIBER CROSS-SECTIONAL AREA RESULTS

	C1	E1	C2	E2
Total	397	336	404	375
Anterior	354	362	365	376
Posterior	471	314	454	373
Superficial	398	368	411	408
Deep	395	306	397	349

	E1/C1	E2/C2
Total	85%	93%
Anterior	102%	103%
Posterior	67%	82%
Superficial	92%	99%
Deep	77%	88%

	Average	Std Dev	P
Total	89%	6%	>0.05
Anterior	103%	1%	>0.05
Posterior	75%	11%	>0.05
Superficial	96%	5%	>0.05
Deep	83%	7%	>0.05

C = control, E = experimental, 1 = Pig B, 2 = Pig C

E.4.2 Fiber Number

The experimental side had a 16% increase in fiber count (SD = 14%, P>0.05) versus the contralateral control side. A comparison between anterior and posterior fibers showed no change in the anterior count, but a 37% increase in the posterior fiber count (SD = 33%, P>0.05). A comparison between superficial and deep fibers showed a 6% increase in superficial fibers (SD = 2%, P>0.05) and a 25% increase in the deep fibers (SD = 26%, P>0.05). None of these values were significant either, due to insufficient sample size.

Table 5: MUSCLE FIBER COUNT RESULTS

	C1	E1	C2	E2
Total	1001	1257	1033	1095
Anterior	289	291	276	276
Posterior	207	334	239	272
Superficial	255	272	243	253
Deep	251	361	275	294

	E1/C1	E2/C2
Total	126%	106%
Anterior	101%	100%
Posterior	161%	114%
Superficial	107%	104%
Deep	144%	107%

	Average	Std Dev	P
Total	116%	14%	>0.05
Anterior	100%	0%	>0.05
Posterior	138%	34%	>0.05
Superficial	105%	2%	>0.05
Deep	125%	26%	>0.05

E.4.3 Fiber Type

A total of 3,014 experimental and 2,923 contralateral control fibers were typed. Table 6 summarizes the ratios and differences in fiber type between the experimental and control side. Comparisons between anterior and posterior fibers and between superficial and deep fibers are also listed. None of these values were significant due to insufficient sample size.

Table 6: FIBER TYPE RESULTS

(C = control, E = experimental)

	Type I	SD	Type IM	SD	Type II	SD	(Type IIA	SD	Type IIB	SD	Type IIC)
C Average											
Total	8.6%	1.3%	21.7%	0.3%	69.7%	1.6%	60.1%	4.8%	9.7%	6.4%	0.0%
Anterior	8.3%	2.4%	24.8%	2.5%	66.9%	5.0%	60.3%	2.6%	6.6%	2.3%	0.0%
Posterior	8.7%	0.4%	16.3%	4.7%	75.1%	5.1%	61.7%	16.5%	13.5%	11.4%	0.0%
Superficial	6.3%	0.7%	19.3%	2.4%	74.5%	1.8%	61.0%	11.5%	13.5%	9.8%	0.0%
Deep	11.1%	2.1%	24.5%	3.3%	64.6%	5.4%	59.0%	2.5%	5.6%	2.9%	0.0%
E Average											
Total	10.5%	1.6%	17.1%	0.4%	72.5%	1.2%	62.0%	6.6%	9.5%	6.9%	0.0%
Anterior	11.4%	4.2%	19.0%	5.7%	69.7%	9.8%	58.6%	18.7%	11.1%	8.9%	0.0%
Posterior	10.1%	0.8%	16.4%	6.4%	73.6%	7.3%	65.6%	2.1%	8.1%	5.2%	0.0%
Superficial	7.7%	1.3%	18.0%	4.4%	74.4%	5.7%	68.7%	6.5%	5.7%	0.8%	0.0%
Deep	13.2%	2.2%	16.0%	4.9%	71.0%	2.8%	57.6%	10.2%	13.4%	12.9%	0.0%
Difference											
Total	1.9%		-4.6%		2.7%		1.9%		-0.2%		0.0%
Anterior	3.1%		-5.8%		2.8%		-1.7%		4.5%		0.0%
Posterior	1.5%		0.1%		-1.6%		3.9%		-5.4%		0.0%
Superficial	1.4%		-1.3%		-0.1%		7.8%		-7.8%		0.0%
Deep	2.1%		-8.5%		6.4%		-1.3%		7.8%		0.0%
% Change											
Total	21.5%		-21.2%		3.9%		3.2%		-2.1%		0.0%
Anterior	36.7%		-23.4%		4.2%		-2.8%		68.2%		0.0%
Posterior	16.8%		0.6%		-2.1%		6.3%		-39.8%		0.0%
Superficial	21.4%		-6.7%		-0.1%		12.7%		-58.0%		0.0%
Deep	19.0%		-34.8%		9.9%		-2.3%		140.5%		0.0%

E.4.4 Capillary Density

The lectin-binding reaction used to identify capillaries was largely unsuccessful. Adequate capillary staining was observed in some sections (Figure 44), but the majority of the sections demonstrated a high degree of non-specific connective tissue staining, making capillary identification nearly impossible (Figure 45).

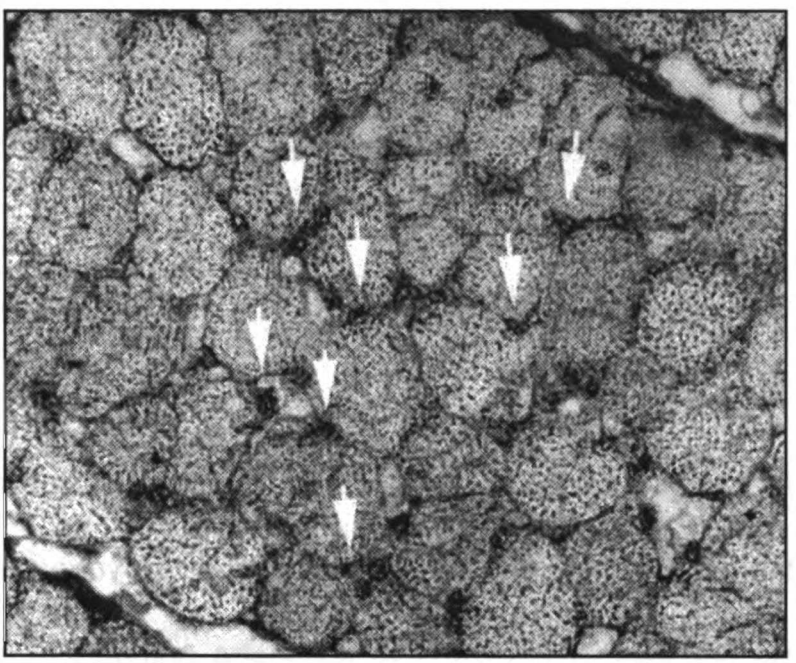


Figure 44: Adequate capillary staining (capillaries indicated by white arrows). x100.

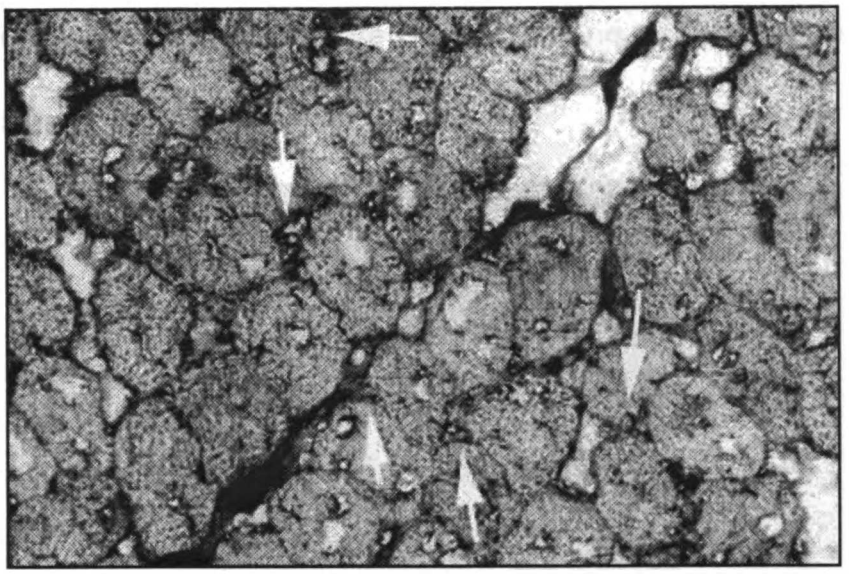


Figure 45: Difficult visualization of capillaries (arrows) due to strong connective tissue staining. x100.

F. Discussion

F.1 Device Failure

As an animal model, the minipig was appropriate for this study. Post-surgical recovery time was remarkable, and device activations were simple. Obtaining adequate device fixation was the greatest challenge in this project. Sibling separation, window-screen and plexiglass cage lining, and infection management all helped reduce device instability. But the most significant factor appeared to be a "lever effect" which occurred whenever the animal placed pressure on the activation arm (L, Figure 46). Force exerted on the lever arm

caused the lower pins to separate from the bone, while the upper pins (F) served as a fulcrum.

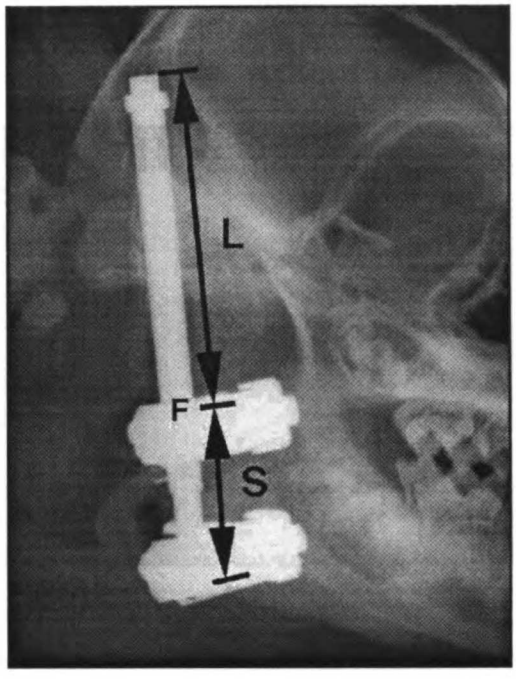


Figure 46: Lever components of the distractor device. L = lever arm, S = support arm, F = fulcrum point. Total length = L+S.

If this theory is correct, the number of days-to-failure should approach zero as the lever length/total length ratio [R= L/(L+S)] approaches 1. Likewise, as the lever length approaches zero, the number of days to failure (DTF) should approach infinity. A Spearman Rank correlation analysis of the negative logarithm of lever-length to total-length ratio versus days to complete device failure (computed on StatView v4.5; Abacus Concepts, Berkeley CA) suggests such a trend exists (P=.06) and that increasing the distance between the support

legs (*i.e.,* maximizing S) would reduce displacement forces exerted on the lower pins.

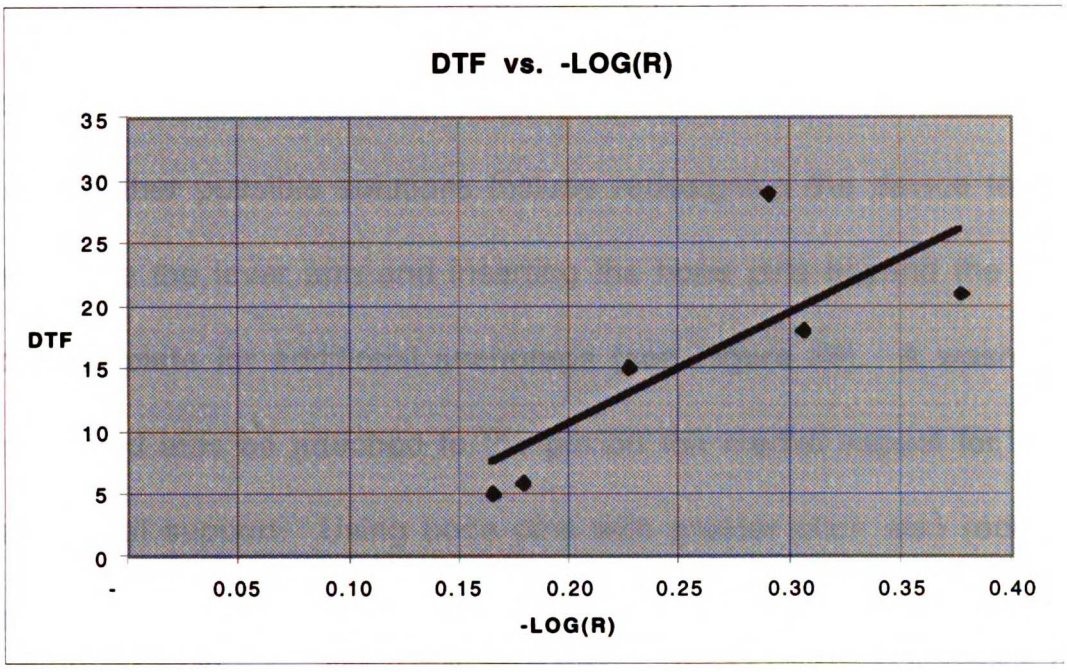


Figure 47: Correlation plot of -log(lever arm ratio) vs. days to failure (DTF). P= 0.06.

ANIMAL	LEVER (L)	SUPPORT (S)	TOTAL (L+S)	RATIO (R)	-LOG(R)	DAYS TO FAILURE (DTF)
A	34	47	81	0.42	0.38	21
B	39	40	79	0.49	0.31	18
C	41	39	80	0.51	0.29	29
D	48	33	81	0.59	0.23	15
E	56	26	82	0.68	0.17	5
G	55	28	83	0.66	0.18	6

Table 7: Lever arm ratios.

Use of an intraoral device could eliminate the problem of leveraging all together. Holzhauer has reported using a Hyrax-based intraoral distraction device in minipigs. In this study, device separation

was not a problem. However, mechanical failure did occur due to eccentric forces related to porcine feeding behavior. The inconvenience of multiple sedations required for device activation was also a concern.[26]

Other possible solutions include redesigning the device to eliminate the lever arm and inserting the bone pins beyond the medial cortical plate for additional anchorage (see Figure 48). A washer and nut could also be attached to the pin on the medial aspect for additional support. Using bone pins with greater pitch also reduces the chance of stripping the pin hole thread. An osseointegrated implant-anchored distractor device such as the one reported by Sawaki *et. al.* could also be helpful.[48] Titanium implants could be placed prior to the osteotomy surgery to ensure adequate osseointegration.

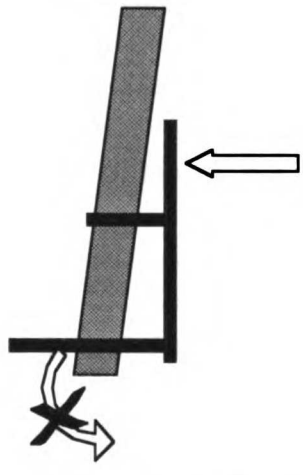


Figure 48: Effect of pin length on device retention. Longer lower pins of distractor (black) may help lock device into bone (gray).

Radiographs and bite manipulations were also relatively simple. However, a radiographic head holder would have been beneficial, since a large variation in angulation was noted in the position of the animals' heads, despite careful alignment and use of support cushions. A holder would have also eliminated the magnification error introduced when the devices moved closer to the x-ray source as the heads grew wider.

The steel implants were useful for determining the actual amount of vertical separation, especially since differential separation between the anterior and posterior ramus occurred on at least two of the animals (A,D). The uneven distraction suggests an equilibrium exists between the theoretical mechanics of the device and the biological effects of perioral musculature, which makes prediction of treatment outcome much more complicated. Placement of anterior implant markers would have been useful to quantify the dimensional differences between the anterior and posterior ramus.

Anterior-facing distractors allowed for better visualization of the distraction site than posterior-facing distractors did. To overcome visual obstruction, a radiolucent distractor device would be ideal. The recent advent of bioresorbable polysaccharide-based

surgical bone plates and screws suggests that this may be possible in the near future.

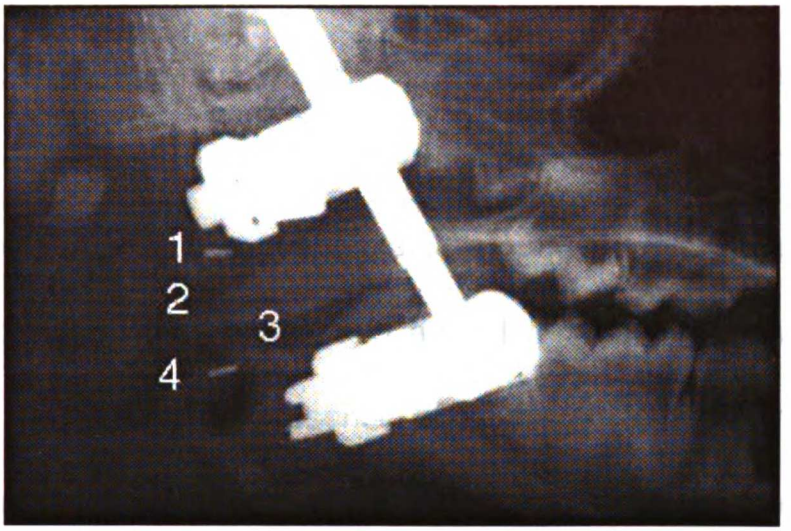


Figure 49: Anterior-facing distractor device. 1,4 = steel implants, 2 = osteotomy, 3 = airway

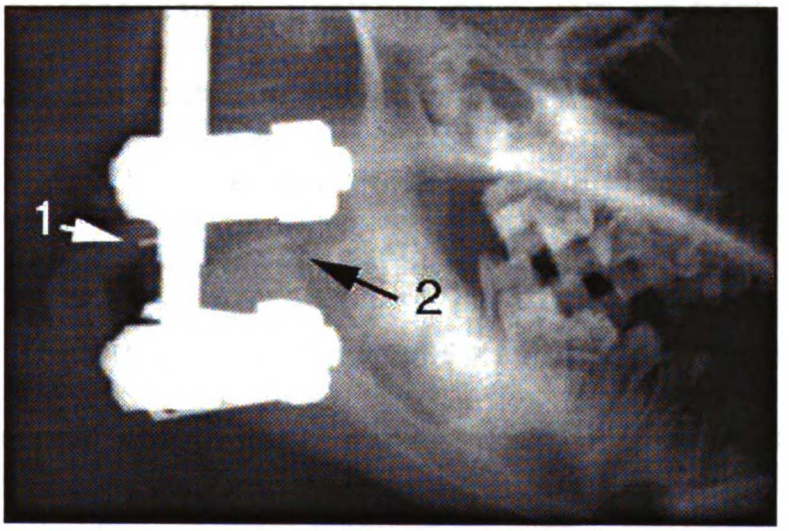


Figure 50 : Posterior-facing distractor device. 1 = steel implant, 2 = osteotomy.

F.2 Bony Changes

The hyperplastic response of the bone was unexpected and is interesting to note in that these changes were not seen on any of the radiographs. Whether this response was a result of the distraction procedure per se or due to other unidentified causes is unknown.

The presence of bony hyperplasia around the pin sites suggests that bone formation may have been an inflammatory response to the "jiggling" of the pins. The presence of acute and chronic inflammatory cells, with increased fibroconnective tissue has been reported at mobile screw sites on miniature pigs that underwent mandibular distraction osteogenesis.[26] Bacterial insult from pin-tract infections may have also contributed to the inflammatory response.

Even though the intended direction of distraction was primarily vertical, the actual direction of distraction was also horizontal and transverse. The broad range of dimensional changes suggests that precise directional control using a unidirectional device is extremely difficult, even under controlled conditions. This observation may be clinically important as even small skeletal changes can profoundly effect both occlusion and facial aesthetics.

The implant markers indicate that the distracted bone is not prone to vertical relapse when consolidated. However, the ramus is prone to narrowing. A significant reduction in ramus width was observed, which can be attributed to four possible causes. The first explanation is an interruption of normal, growth-related remodeling. As the mandible develops, bone is deposited along the posterior border and resorbed along the anterior border. If the apposition process is disrupted and anterior resorption continues, this would result in a narrow ramus. Either surgical trauma or the distraction could have caused disruption of the remodeling process. The biology of such a mechanism (if one exists) is unclear.

The second explanation is that posterior-border bone resorption from increased muscle tension secondary to surgical trauma caused ramus narrowing. This explanation is supported by the observation that narrow rami were noted even when very little distraction was achieved (Pigs F,G). Muscle contraction from scar formation could have increased tension on the posterior and inferior mandibular borders, leading to increased resorption. The apposition process did not have to be disrupted per se; it just had to be not enough to overcome the rate of resorption.

The third possibility is that the distraction procedure stretched the muscles, and the increased tension on the mandible resulted in accelerated bone resorption at the muscle attachments. The fan-shape of the masseter muscle of the pig has been well-documented by Herring.[23,24] The attachment of the muscle corresponds to the areas where ramus width appears to have been affected, making this theory plausible. (As a clinical aside, the human masseter has no dorsally attaching fibers, so if this theory is correct, posterior bony resorption should not be seen in human distraction, but inferior border resorption might.)

The fourth hypothesis is that the horizontal component of the distraction pushed the mandible up against the surrounding tissues, and the pressure from this impingement triggered accelerated anterior border resorption above and beyond the normal rate. A comparison of vertical distraction versus width reduction does not support this explanation. If this were the case, one would expect to see decreased width reduction with increased vertical distraction. The data suggest the opposite, and the greater the vertical distraction, the greater the width reduction. This theory could be readily tested by placing implant

markers along the anterior border to monitor the rate of resorption with and without distraction.

ANIMAL	VERTICAL DISTRACTION	WIDTH REDUCTION
B	33%	7%
C	80%	22%
D	56%	23%

Table 8: Comparison of vertical distraction to ramus width reduction

F.3 Muscle Changes

Histologically, an increase in the number of fibers was observed in the posterior (37%), deep (25%), and superior (6%) regions of the masseter. No change in fiber number was observed in the anterior masseter. Even though these results were not significant, they were consistent with the proposed hypothesis. The difference in response between the anterior and posterior can be attributed to the unequal stretching observed. The posterior fibers were stretched more than the anterior fibers, so a greater amount of proliferation in the posterior would be expected. The difference between the superficial and deep fibers is consistent with Alway's theory that the pattern of stretch-induced muscle fiber formation is type specific. His data indicate that new type I fiber formation occurs more consistently than

new type II fiber formation in response to chronic stretch.[2] Because there were more type I fibers in the deep masseter (approximately 70% more than in the superficial masseter), greater fiber proliferation would be expected in the deep regions.

The mean fiber cross-sectional area was reduced in the posterior, superficial, and deep regions, with a mild increase in the anterior fiber area. While these findings were not significant, the trend was consistent with the hypothesis. The posterior fibers, being more perpendicular to the tension would have led to fiber atrophy. The anterior fibers, being more parallel to the stretching, would have led to fiber hypertrophy. Fiber-area reduction was observed in both superficial and deep regions due to greater perpendicular components in each of these areas.

The effect of disuse atrophy is unknown, but any contribution was probably minimal since a change in the functional habits of the animals was not observed. The pigs, being pigs, appeared to function quite well on both experimental and control sides during eating.

The results from fiber typing were not significant, but consistent with the published reports by Suzuki,[58] Strohm and Holm,[56] and Tuxen.[60] Suzuki reported that from age 4 weeks to age 8 weeks,

the pig masseter demonstrates an increase in type I fibers from 10 to 15%, a decrease in intermediate (IM) fibers from 7 to 5%, and a decrease in type II fibers from 83 to 80%. According to Tuxen, the pig masseter is comprised of 77.6% type II, 20.7% type I, and 4.3 % type IM fibers. According to Strohm and Holm, 68-87% of the pig masseter fibers are type II.

The fiber type proportions in this study were similar to Suzuki's results for type I identification (9%), but less so for type II (70%) and IM fibers (21%), due to difficulty in determining the difference between "moderately" stained (IM) and "darkly" stained (II) fibers. The large difference observed between superficial and deep type I percentages (control = 6.3% superficial, 11.1% deep; experimental = 7.7% superficial, 13.2% deep) suggests that sampling both areas independently was appropriate.

The greatest percent increase was observed in type I fibers, which is consistent with results from chronic-stretch studies on avian ALD muscle fibers[1,2] and simian anterior digastric fibers.[7] Each of these studies reported an increase in type I fibers in response to chronic stretch. In this study, growth was a confounding factor, since type I fibers in the mandible naturally increase with growth. Any

definitive change would have to be above and beyond increases attributable to growth.

F.4 Conclusion

The preliminary data from this study suggest that the proposed hypothesis cannot be rejected. Further studies are required to confer significance since this study had insufficient sample size. The presence of irregular bony formation, decreased ramus width, and muscle scarring, are all noteworthy clinical observations worth further investigation, especially since device failure is also a common complication associated with distraction osteogenesis of the human mandible. The precision and predictability of skeletal dimensional changes when using a unidirectional distractor device also warrants further scientific investigation. The information gathered from this project should be useful for future studies utilizing the minipig model to investigate the biological effects of distraction osteogenesis.

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