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Histological Characterization of the Exercise-Dependent Alterations in the Musculoskeletal System of Zebrafish

Dissertation

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Dedicated to

my beloved mother and father

Abstract

Gain in bone mass is facilitated by physical exercise through adaptive responses of the musculoskeletal system. Specific types of physical exercise can increase muscle and bone strength, body coordination, and balance. More importantly it leads to measurable fracture risk reduction in humans. Zebrafish have been found to represent an advantageous model to study bone as well as muscle modeling and mechanical adaptations in response to physical exercise during early developmental stages. To test the hypothesis that musculoskeletal exercise causes bone and muscle adaptation in adult zebrafish, and to characterize those adaptations, zebrafish were subjected to increased physical exercise for four weeks in a swim tunnel experiment and compared to a non-exercise group.

The goal of this study is to quantify the cellular, compositional and structural adaptations of the musculoskeletal system of zebrafish. Four weeks of physical exercise promoted bone adaptations with increased numbers of osteoblast and osteoblast related parameters, including number of osteoblasts per bone perimeter (N.Ob/B.Pm), and osteoblast surface per bone surface (Ob.s/BS) in the exercise group. However, decreased osteoid thickness (O.Th) and osteoid surface per bone surface (OS/BS) were detected in exercised group. The bone volume per total volume (BV/TV) and the number of osteocyte per bone area (N.Ot/B.Ar) were higher in the exercise group compared to the control group. Regarding muscle adaptations, higher numbers of muscle fibers and centrally nucleated muscle fibers were found in the exercise group. However, the size of muscle fibers was much smaller in the exercise group.

The results of this study provide new information on the influence of physical exercise on the musculoskeletal system of zebrafish, which is an important animal model in the field of musculoskeletal research.

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1 Introduction: The musculoskeletal system

Muscle and bone are inextricably associated by common genes regulating body size, by their physical connection, and by a shared mechanical loading environment [1]. It is assumed that increased muscle mass and strength result in a corresponding increase in bone mass and strength [2] [3], and that both tissues grow proportionally to each other, because bone is biomechanically linked to muscle [4]. Muscles become larger and stronger in response to increased exercise, while bone adapts to increased forces imparted by muscles during exercise via adding size, mass, and strength [5]. Muscle force is a strong determinant of bone mass and strength. Bone is adapted to the tissue strain due to biomechanical forces. This process is modified by hormonal signals, such as estrogens and androgens [6]. The intimate relationship between bone and muscle propose the question that a quantitative expression describing the relationship between muscle force and bone mass could be a useful functional unit [3]. According to this consideration, the functional “muscle - bone unit” was introduced to the literature [7] [8] [9]. Consequently, as muscles become larger and stronger in response to increased loading or exercise [10], the concept of a functional “muscle - bone unit” is supported by the biomechanical link between muscle and bone, in which changes in muscle mass and strength should also affect bone mass, size, and strength predictably and correspondingly [3] [11].

This chapter first gives an introduction into function and composition of bone tissue with focus on mechanosensitivity, secondly into the function and composition of muscle with focus on muscle adaptations, and thirdly it provides the aim of this thesis.

1.1 Endoskeletal bone

Bone has diverse mechanical, biological, and chemical functions, such as structural support of the body, protection of internal organs, production of growth factors. Further bone plays a central role in the maintenance of mineral homeostasis [14]. The mechanical environment plays an important role in the regulation of bone remodeling in intact bone and of modeling during bone repair [12]. Bone cells are capable of sensing and responding to mechanical forces, which is referred to as

mechanosensitivity. Various mechanical stimuli revealed different effects on bone cells, because loading and the involvement of numerous signals are sensed and transduced [12].

1.1.1 Bone mechanosensitivity

Vertebrate bone is a metabolically active tissue and bone mass is balanced through the activity of osseous cells called osteoblasts, i.e. bone-forming cells, and osteoclasts, i.e. bone resorbing cells [13]. Cells of a third type called osteocytes are former osteoblasts that became entrapped inside the bone matrix [15]. In humans, they are the most abundant bone cell and represent approximately 90–95% of all osseous cells in the adult skeleton [16]. Osteocytes are found in cavities in the bone matrix called lacunae and are separated from direct contact with the mineralized bone matrix by a thin layer of unmineralized tissue [17] [18]. It has been demonstrated that osteocytes act as mechanosensors of bone. They are thought to be mechanosensory cells which coordinate the activity of osteoblasts and osteoclasts in bone repair and bone renewal by detecting mechanical stimulation and organizing signal transmission between bone cells [18] [19] (Figure 1). Bone modeling / remodeling and mineral homeostasis are regulated by osteocytes in response to shear or strain forces [20]-[22] under hormonal, neural, immunological, and mechanical control [23].

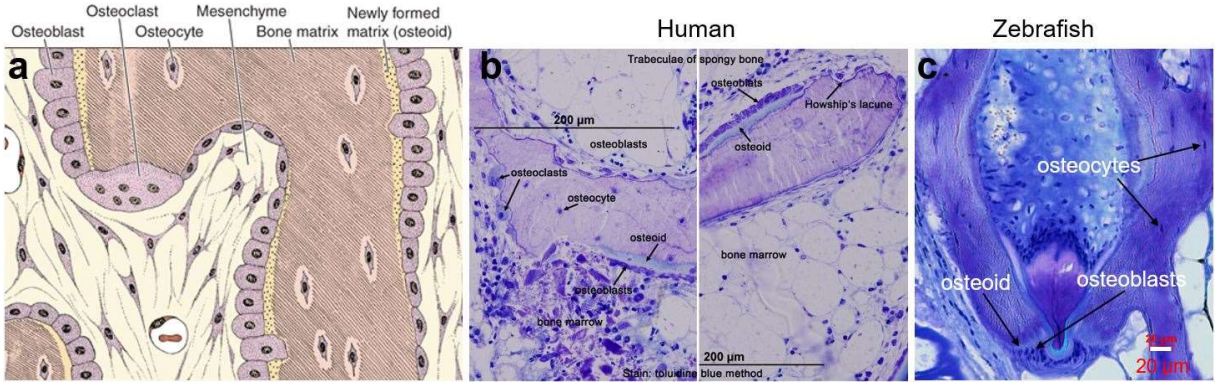

Figure 1: (a) Schematic showing osteoblasts, osteoid, osteoclasts and osteocytes. Bone tissue is continuously remodeled through the actions of bone cells, which include bone resorption by osteoclasts and bone formation by osteoblasts. Osteocytes work as mechanosensors and orchestrators of the bone remodeling process. Osteoblasts dominate the process of forming bone tissue by secreting the osteoid which is the unmineralized, organic portion of the bone matrix ((a) is from [24]). (b) Toluidine blue staining trabeculae of human spongy bone ((b) is from [25]). (c) Toluidine blue staining vertebrate bone of zebrafish. Osteocytes are embedded in the calcified bone matrix.

1.1.2 Mechanical loading and bone modeling

Mechanical loading is a key factor in the differentiation of cells and tissues, it engages in differentiation and morphogenesis from the level of individual cells to whole organism patterning [26]. It also plays an important role in the development and maintenance of vertebrate tissues [27] [28]. Especially in muscle, bone, and other connective tissues, mechanical loading is considered to modulate the genetically determined tissue and body architecture [29]. Mechanical loading is also playing a fundamental role in bone health, and reduced mechanical loading is associated with bone loss [30]. Mechanical loading in bone is transformed through osteocytes which sense and transform the mechanical stimulus into biological signals. The structure of the lacunar-canalicular network determines the interconnectivity of osteocytes and thus their capability to sense mechanical loading and send biological signals to regulate bone remodeling regarding both bone resorption and formation [17] [31]. Bone remodeling aims to repair fatigue damage, to prevent excessive aging of bone material, and its consequences [32].

Skeletal aging is associated with a decrease in osteoblast activity and an increase in osteoclast activity [35] which leads to an imbalance between bone resorption and bone formation. Furthermore, aging causes common skeletal diseases like osteoporosis by reducing the amount of bone tissue. Aging was associated with a lower loading-induced increase in osteoblast number on the periosteal surface [36]. Bone loss is also linked to reduced osteoblast and impaired osteocyte functions. Physical exercise, which is the main method to improve bone strength and maintain bone mass later in life, has been recommended as a preventive and therapeutic strategy against aging-induced bone weakness and associated fractures [37]. Loading increases trabecular thickness and the number of trabecular connections [36]. Bone modeling contributes to skeletal growth and is activated in response to excessive loading of bone, leading to bone formation [33]. To investigate the role of mechanical loading on skeletal tissue, several different ways could be considered. Endurance training is considered one of the common and effective ways to impose increased mechanical loading [34]. In this context, fish have several advantages over mammalian models. For example, fish could control buoyancy by altering their swim bladder and the influence of gravitational forces are relatively limited compared to terrestrial

animals [34]. As a consequence, the forces generated by the fish and the reactions of the surrounding medium can directly arise the main loads on the tissue. Moreover, fish have relatively simple locomotory musculature [34]. Particularly zebrafish offer several advantages over mammalian models to investigate the effects of increased mechanical load on development of muscle and bone [38].

1.2 Skeletal muscle

Skeletal muscle is one of the most dynamic tissues of the human body with remarkable adaptive capabilities, comprising approximately 40 % of total body weight and 50–75 % of all body proteins [39]. The main functions of skeletal muscle are the control of movement and posture and to influence the whole body's energy metabolism.

1.2.1 Muscle fiber types

Skeletal muscle is mainly composed of myocytes, adipocytes, and fibroblasts [40] (Figure 2). Myocytes also referred to as myofibers (muscle fibers) which are multinucleated and post-mitotic. Muscle function is characterized by muscle structure and by the composition of different fiber types. Muscle fibers can be assigned to different fiber types, with characteristic movement rates, response to neural inputs, and metabolic styles [41] [42]. Specific fiber types are a feature of vertebrate muscle; for example, adult mouse and fish musculature indicates a gradation of myosins [43] [44], patterns of innervation [45], metabolic activity [46] [47], and many other distinguishing characteristics [41] [42] [48].

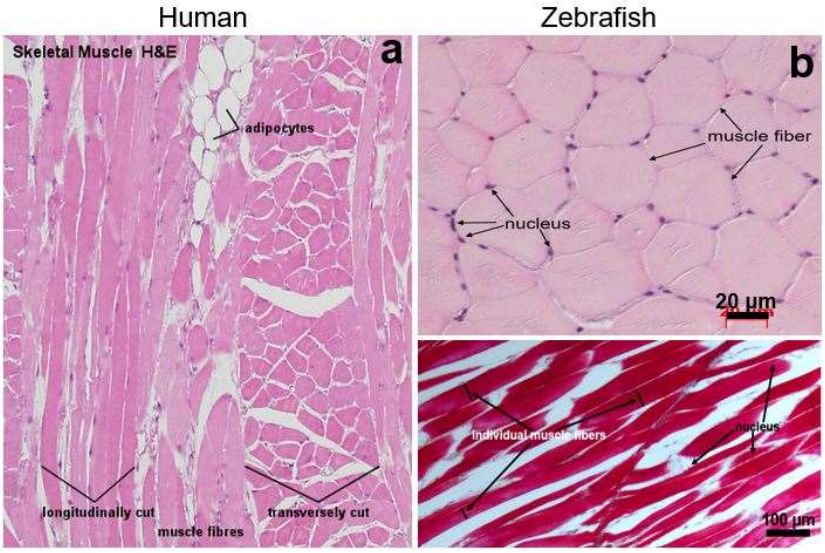

Figure 2: (a) H&E staining of human skeletal muscle fibers ((a) is from [49]). Both longitudinally and transversely cut muscle fibers are present. (b) H&E staining of muscle fibers in zebrafish cross-sectional view. As the picture shows skeletal muscle is mainly composed of myocytes that develop from myoblasts. Myocytes are multinuclear, post-mitotic cells. (c) Longitudinal section of muscle fibers in zebrafish. Skeletal muscle fibers (individual cells) are long and cylindrical cells, with multiple nuclei that are located on the perimeter of the fibers. Myoblasts differentiate, align and fuse together to make longer, multinucleated tubes called myotubes. New muscle fibers are proliferated and produced from satellite cells following muscle injury.

Skeletal muscle fibers are classified as "slow-twitch" (type 1) and "fast-twitch" (type 2), according to differential gene expression of myosin heavy chain (MYH) and metabolic activity, and there is further classification of fast-twitch fibers into three major subtypes, including types 2A, 2X, and 2B [50] [51] (Figure 3). Skeletal muscle fibers also differ in energy production. Type 1 and 2A fibers primarily make use of oxidative metabolism, while type 2X and 2B fibers primarily rely on glycolytic metabolism [51]. The fast-twitch fibers take a shorter time to reach peak force and can generate higher amounts of force, but they are quicker to fatigue when compared to slow-twitch fibers. The structure of skeletal muscle is determined by a well-described arrangement of muscle fibers and associated connective tissue [39]. The number, size and type of individual muscle fibers determine the size and strength of muscle, however, pathological infiltration by fat and connective tissue may change this relationship [52] [53].

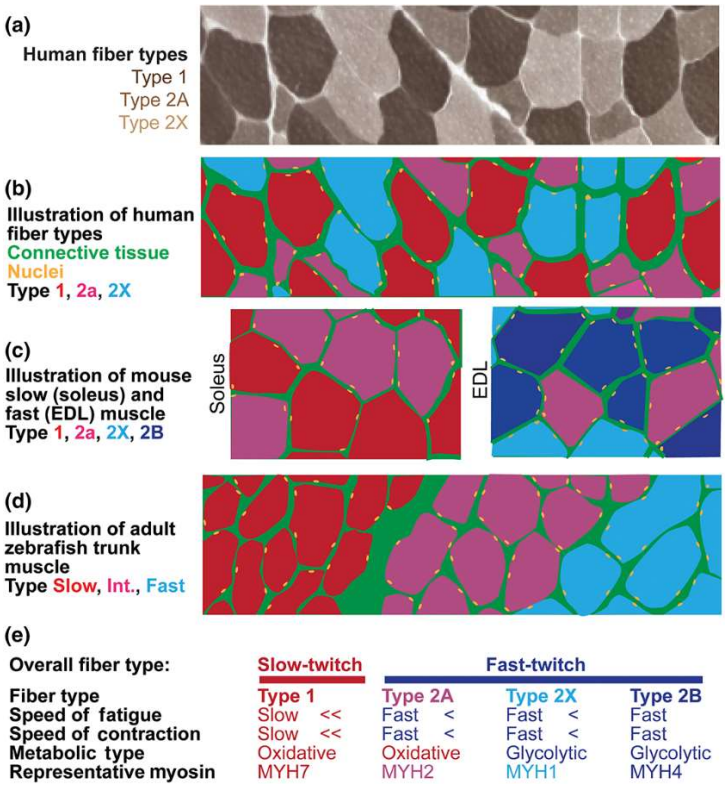

Figure 3: Skeletal muscle fiber types. (a) Section of human muscle, which shows fiber types have been differentiated using ATPase staining after pre-incubation at pH 4.6. (b) Illustration showing healthy muscle fibers. Connective tissue (green) and muscle fiber nuclei (orange) are shown. Different fiber types, including type 1 (red), type 2A (pink), and type 2X (light blue), can be intermingled within a single mammalian muscle. (c) Particular muscle groups can also be enriched for slow (Soleus) or fast (EDL: extensor digitorus longus) muscle fibers. In mouse, an additional fast fiber type, 2B (dark blue), is present. (d) In zebrafish trunk musculature, different fiber types are segregated, with the slowest fibers discovered laterally, and fast fibers discovered medially. (e) Crucial properties of fiber types, with the color code highlighting the graded shift from slow to fastest fibers. Muscle fibers types are defined by their myosin heavy chain (MYH) expression. There are also many other factors which can distinguish fiber types. For example, metabolic programs also contribute to muscle fiber phenotype. Figure 3 is from [51].

1.2.2 Physical exercise and muscle adaptation

Physical exercise provides multiple beneficial effects upon skeletal muscle [54] and strong beneficial effects on health by modifying metabolic potential, morphology, and physiology of skeletal muscle [54] [55]. Adult skeletal muscle regenerates after exercise, muscle injury, or degeneration [56]. Moreover, physical exercise causes various muscle adaptations and regenerative responses, which include most importantly the modification of the size, structure, number, and type of muscle fibers [57]. The function of muscle fibers is also influenced by changing their structure and metabolism and contributing to release of growth factors and some signaling molecules

[58]. Many growth factors are generated in injured skeletal muscle and influence its regeneration process [59].

Physical exercise influences the growth of muscle in several ways. Firstly, it modifies the number of muscle fibers which takes place by splitting of myofibrils. Secondly, it modifies the diameter of muscle fibers. Thirdly, it modifies fiber length [60]. Growth of muscle takes place by hypertrophy of the existing muscle fibers through adding new myofibrils or by adding new sarcomeres to the ends of the existing muscle fibers to increase their length [60].

Regarding the modification of the number of fibers in response to exercise, it was shown that exercise caused muscle damage [61]. This damage activates the process of muscle repair in terms of activating satellite cells towards the regeneration of muscle fibers. Indeed, satellite cells play an important role in the regeneration and growth of muscle fibers. They are located on the external surface of muscle fibers and closely related to muscle fibers [62]. The satellite cells start replicating during the repair of injured muscle fibers and during the growth of muscle fibers. The quiescent satellite cells are activated by e.g. increased physical exercise, muscle fiber injury and increased muscle tension (Figure 4). During this process skeletal muscle becomes more responsive to training and regeneration. Literature indicates that the number of satellite cells increases in response to short- [63] [64] and long-term [65] [66] [67] resistance training. Additionally, some studies showed that there are increased numbers of satellite cells accumulated in the muscle tissue of power lifters [68]. Regarding the modification of the number of fibers, endurance exercise, for example swimming or running, gradually causes a transformation of type II B fibers into type II A fibers. The transformed muscle fibers show changes in diameter, mitochondria, blood capillaries and strength [69]. Although endurance exercise does not contribute to muscle mass [70], it can result in adaptations of the cardiovascular and respiratory system, which in turn may cause skeletal muscle to receive better supplies of oxygen and carbohydrates [71].

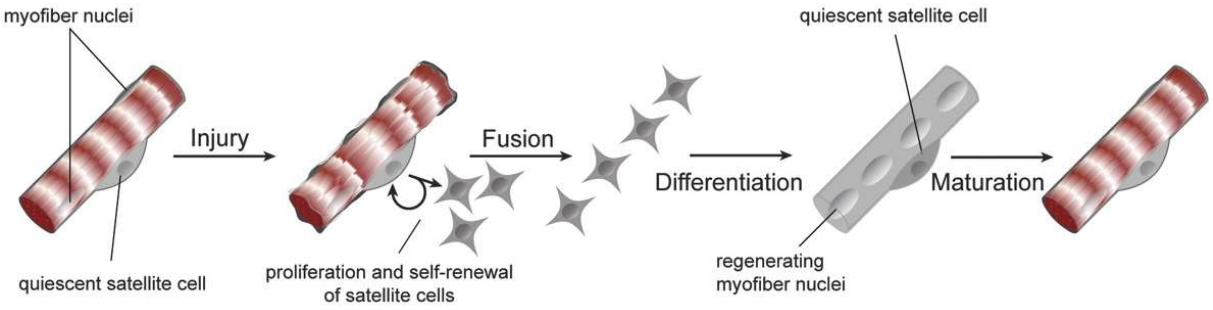

Figure 4: Satellite cells are muscle-resident stem cells and they are located underneath the basal laminae of myofibers, however, they are normally quiescent. In response to muscle injury, the quiescent satellite cell is activated, starts to proliferate as myoblasts, fuses and differentiates into myotubes which will grow replacing damaged muscle. Satellite cells happen self-renewal and proliferate to generate myoblasts which fuse and form newly regenerated myofibers (characterized by centrally nucleated fibers). This figure is from [72].

Another indicator for newly regenerated muscle fibers during muscle regeneration are centrally nucleated fibers (CNFs), where the myofibers have nuclei in the center of the cytoplasm [73] [74]. Muscle histology is one standard approach to analyze muscle function. H&E staining can be used for the morphological analyses and provide parameters including size, number and diameter of myofiber, the amount of centrally nucleated muscle fibers, the presence of degenerated and regenerated myofibers, and adipocytes and fibrotic cells.

1.3 Motivation and Aim of this study

Zebrafish (*Danio rerio*) are widely accepted as a vertebrate model for genetics, physiology, development, reproduction, disease (immunology, toxicology, oncology) and aging [75] - [79]. There are several advantages for biological studies of zebrafish compared to classical animal models such as rats and mice, including a shorter generation time, smaller body size and higher housing capabilities.

The aim of this study is to test the hypothesis that musculoskeletal exercise causes adaptation of the muscle-bone unit in adult zebrafish. To characterize possible adaptations of the vertebral muscle-bone unit in the zebrafish spine, this study quantifies the cellular, morphological and structural adaptations of musculoskeletal tissue of zebrafish which underwent increased physical exercise for four weeks in a swim tunnel experiment.

2 Materials and Methods

To study the increased musculoskeletal loading in zebrafish, a musculoskeletal exercise regimen was designed and histomorphometric parameters were assessed.

2.1 Animal model

The study was performed in mixed sex adult zebrafish (Figure 5). Twelve zebrafish at an age of 4.5 months were used for the experiment. All zebrafish were raised under the standardized conditions before the experiment according to Jarolim and Lieschke et al. [69] [79]. Animal experiments obtained the approval of the institutional review board and were performed according to ethical guidelines of local committees of Hamburg (No.42/16).

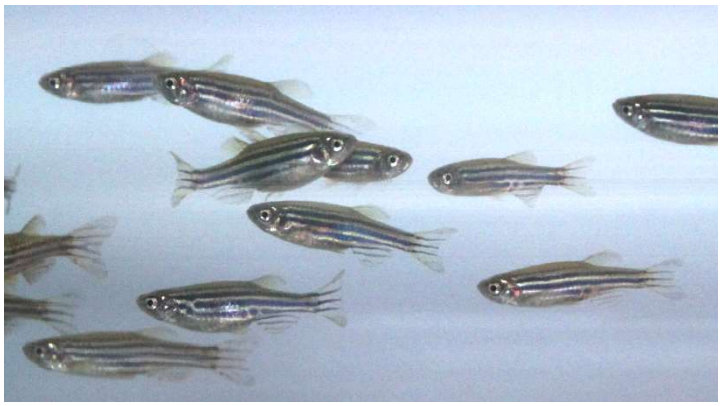

Figure 5: Experimental animal model. Four and a half months-old zebrafish were used in this study and randomly divided into a control group and a physical exercise group. All zebrafish were raised under the same standardized conditions [80].

The zebrafish were randomly divided into a control group and a physical exercise group (n=6 per group). Each group was accommodated in a 60 l water tank with a constant temperature (26°C), water hardness (6-12°dH), pH (7.4), nitrite (<0.3 mg/L) and nitrate (25-100 mg/L) levels. Flake food (Tetramin Tropical Flakes Fish Food, Tetra SpectrumBrands, Blacksburg, STATE USA) was given twice a day. Photoperiod was kept at a 14 h light/10 h dark cycle [81].

2.2 Experimental design

To exercise the zebrafish we used a custom-built swimming tunnel within a 70 l water tank (Figure 6a). A swimming chamber producing a laminar flow was used to

encourage the fish to swim at defined velocities as described in a previous study [70]. Fish from the study group were exercised five days per week for four weeks, where they swam against a flow with a velocity of 12 cm/s (Figure 6b). Any direct illumination of the experimental set-up was avoided to reduce visual disturbances that could affect swimming during the test period. Increasing exercise periods were applied from 4 h in the first week, 5 h in the second and third week, to 6 h in the last week. In contrast, the control group stayed unexercised and with normal swimming behavior in the 60 l water tank during the 4 weeks of the experiment (Table 1).

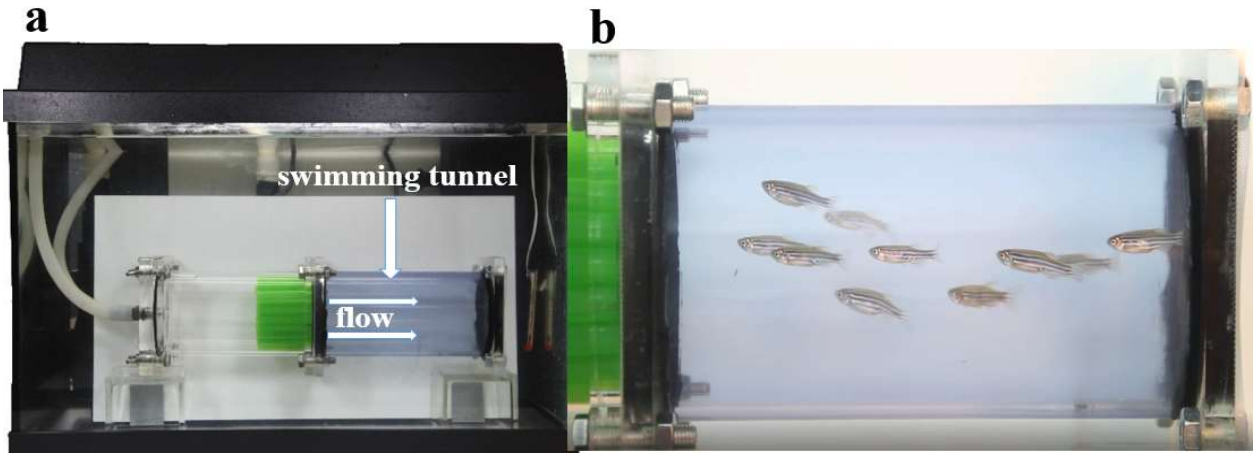

Figure 6: Experimental design. (a) A swimming tunnel was used to perform the exercise. The picture shows the swimming tunnel within a water tank, with a laminar flow that encourages fish to swim at a defined velocity. (b) The exercise group performed increased physical exercise, *i.e.* swam at a velocity of 12 cm/s for 4 h per day in the first week, 5 h per day in the second and third week and 6 hours per day in the last week, as detailed in Table 1. The zebrafish in the control group stayed unexercised with normal swimming behavior in the water tank. All other conditions were the same, such as water temperature, water hardness, pH, nitrite and nitrate levels, fish food, as well as photoperiod.

Table 1: Physical exercise regime in the groups. Outline of increased physical exercise regime in the exercise group.

	Week 1	**Week 2**	**Week 3**	**Week 4**
Control	0h/day	0h/day	0h/day	0h/day
Exercise	4h/day	5h/day	5h/day	6h/day

Three days after the last training, an overdose of tricaine methane sulfonate was used to sacrifice the fish.

2.3 Measurement of macroscopic body changes

The average body weight and body length was measured separately in the exercise group and the control group, to investigate whether possible macroscopic body changes occurred with exercise using a micro-scale and a caliper.

2.4 Sample preparation

The sacrificed zebrafish of the exercise group (n=6) and the control group (n=6) were randomly divided into two groups, respectively, for bone histomorphometry (n=3 per group) and muscle histomorphometry analysis (n=3 per group). Sacrificed fish were prepared by the following steps. First, samples were step-wise dehydrated in an increasing series of alcohol solutions with 80%, 96%, 100% ethanol overnight (Autotechnikon, Enno Vieth Mikrotome GmbH, Wiesmoor, Germany). Samples were placed in infiltration solutions, in infiltration solution #1 for 24 h, in infiltration solution #2 for 5 days. Embedding media was composed of methyl methacrylate (MMA) solution and accelerating solution 2,4,6-Tris(dimethylaminomethyl)phenol (DMP). For preparation of 10 glass jars, 200ml MMA and 1ml DMP (using pipette) were mixed. Samples were transferred from infiltration to embedding media and embedded lengthwise for bone histology and transversally for muscle analysis (Figure. 7a, b). Polymerization took place in a fridge cooled to 4°C. To enable the analysis of bone and muscle histomorphometric parameters, samples were respectively cut longitudinally (Figure 7c) and transversally (Figure 7d) into 4µm thick sections.

2.5 Staining

Masson-Goldner trichrome and Toluidine blue stainings were performed to analyze bone histomorphometry (Figure 7c). Hematoxylin and eosin stainings (H&E stain) were performed for muscle histomorphometry (Figure 7d, Appendix 1, 2, 3). Masson-Goldner trichrome is a three-color staining and used to visualize connective tissues, particularly collagen, in tissue sections. In a standard Masson's Trichrome procedure, collagen is stained blue, nuclei are stained dark brown, muscle tissue is stained red, and cytoplasm is stained pink. Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components. Toluidine blue is used in tissue sections with the aim to highlight components, such as mast cells

granules, mucins, and cartilage. Mastocytes and cartilage is stained purple, mucins is stained purple or red, nuclei is stained blue. Hematoxylin has a deep blue-purple color and stains nucleic acids. Eosin is pink and stains amino acids/proteins nonspecifically. In a typical tissue, nuclei are stained blue and extracellular matrix have varying degrees of pink staining.

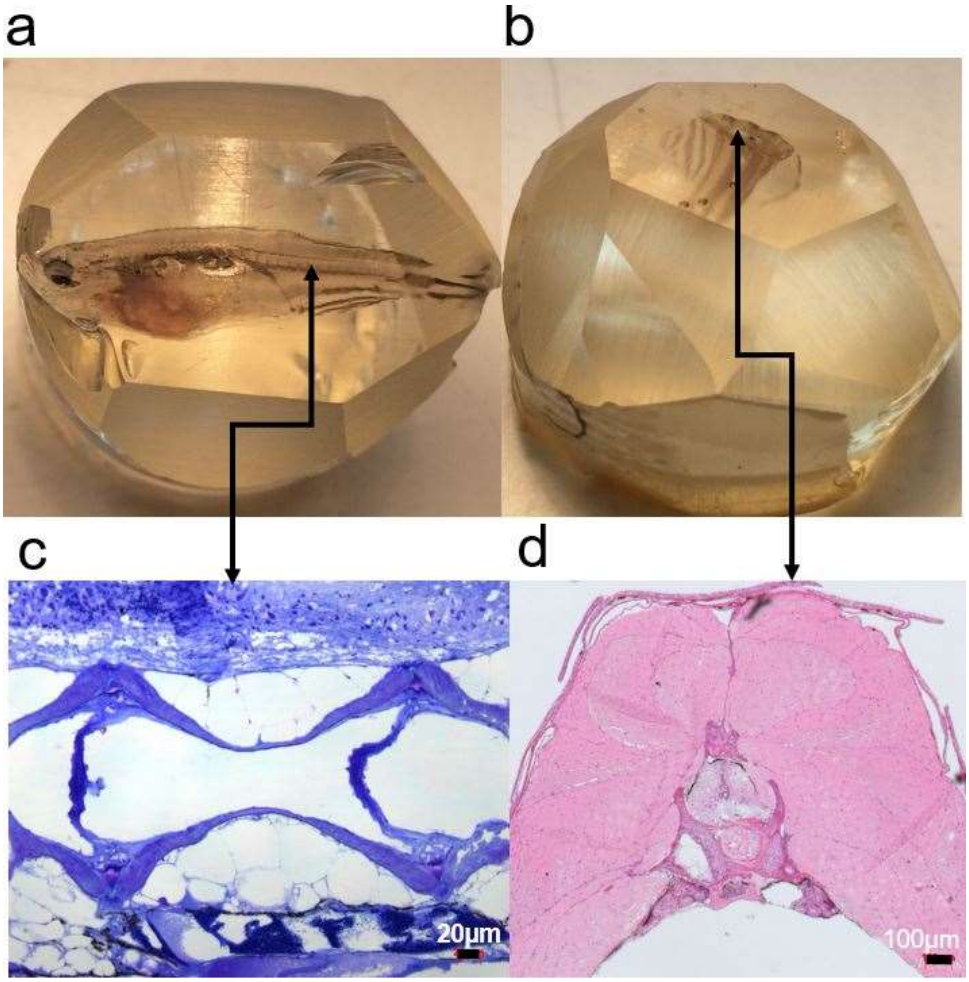

Figure 7: Different embedding and histomorphometry methods applied within this study. The picture illustrates that (a) a longitudinally embedded zebrafish and (b) a transversally embedded zebrafish. (c) Toluidine blue staining displays longitudinal bone histology. (d) Hematoxylin and eosin staining (H&E stain) display the cross section of muscle fibers. Longitudinal embedding was used for bone histology and transversal embedding was used for muscle histology.

2.6 Musculoskeletal Histomorphometry

Bone and muscle histomorphometry were analyzed separately to test bone and muscle adaptation in adult zebrafish caused by musculoskeletal exercise.

2.6.1 Bone Histomorphometry

The OsteoMeasure histomorphometry system (OsteoMetrics, Atlanta, GA, USA) was used to quantify the osteoid and osteoblast related parameters, including number of osteoblasts per bone perimeter (N.Ob/B.Pr, 1/mm), osteoblast surface per bone surface (OB/BS, %), osteoid surface per bone surface (OS/BS, %) and osteoid thickness (O.Th, µm). The Bone Volume over Total Volume (BV/TV) and the osteocyte number per bone area (N. Ot/B.Ar) were also assessed. Eight microscopic fields of each sample section were evaluated at 20-fold magnification. Related parameters were measured in Masson-Goldner trichrome and Toluidine blue stained sections.

2.6.2 Muscle Histomorphometry

Image J (Fiji) which is a public domain Java image processing and analysis program inspired by NIH Image, was used to conduct this analysis after taking high resolution images with the microscope. The muscle analysis focused on number of muscle fibers and muscle fiber size in a certain area. Muscle fiber regeneration was assessed by the percentage of muscle fibers containing central nuclei. Three different anatomical regions along the spine were chosen for the analysis (Figure 8). Each sample section was evaluated at 20-fold magnification. Hematoxylin and eosin staining (H&E stain) was performed for the muscle analysis (Figure 9).

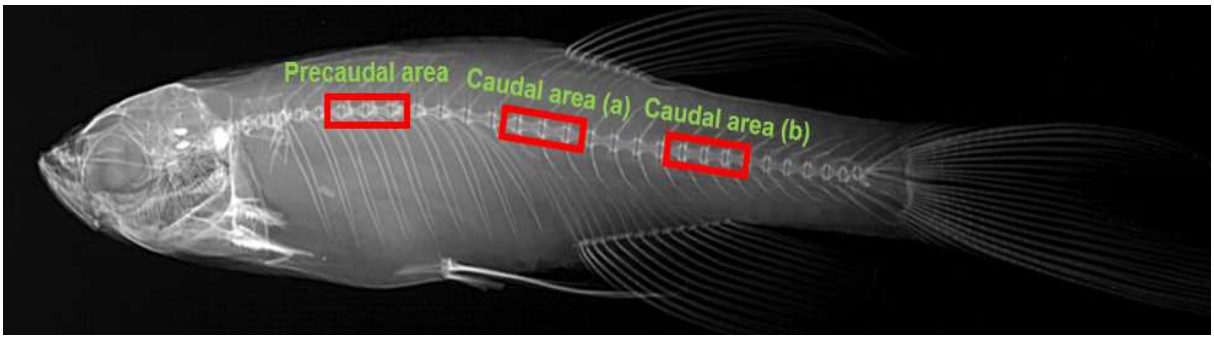

Figure 8: Comparing musculoskeletal differences between the caudal and the precaudal regions that responded to increased swimming exercise. The precaudal vertebral area, caudal vertebral area (a) and caudal vertebral area (b) were analysed regarding musculoskeletal differences after one month increased swimming exercise.

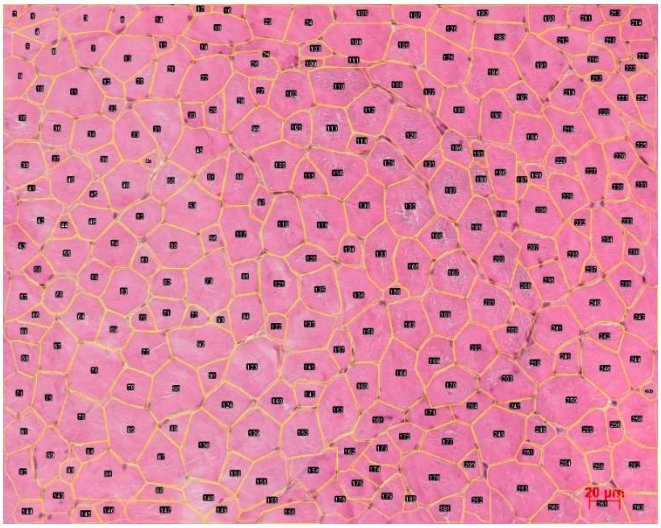

Figure 9: Number of muscle fibers were quantified with Image J (Fiji). The diameter and number of muscle fibers are associated with muscle strength. This figure shows a cross section of muscle fibers, the yellow line is used to measure the area of each muscle fiber, and the black dots indicate the muscle fibers count.

2.6.3 Statistical Analysis

SPSS (Version 24, IBM, Armonk, NY, USA) was used for statistical analysis. Differences between control and exercise groups were compared by the student t-test. Statistical significance was defined as $p<0.05$.

3 Results

The musculoskeletal exercise regime performed on the zebrafish resulted in no differences regarding the macroscopic morphology between the exercise and control group, but it showed clear alterations of bone and muscle tissue histomorphometry (Figure 10). Results are hereinafter described as mean ± standard deviation.

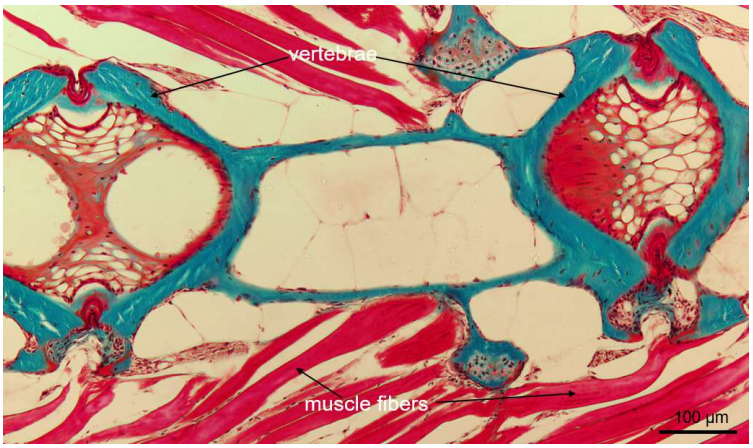

Figure 10: Masson-Goldner trichrome staining of zebrafish vertebral column illustrates the inextricably close relationship between muscle and bone from structural analysis, which have strong physical connections and a shared loading environment. Physical exercise are assumed to cause adaptations in the biomechanical unit of muscle and bone, because both tissues interact mechanically and functionally.

3.1 Macroscopic body changes

The average body weight was slightly lower in the control group compared to the exercise group but did not reach significance. The control and exercise groups weighed 0.27 ± 0.03 g vs. 0.29 ± 0.06 g, respectively (p=0.67). The average body length of control and exercise group were 2.51± 0.38 cm vs. 2.41± 0.52 cm and, respectively, p=0.69 (Figure 11). There were no significant differences in body weight and body length between the control and exercise group.

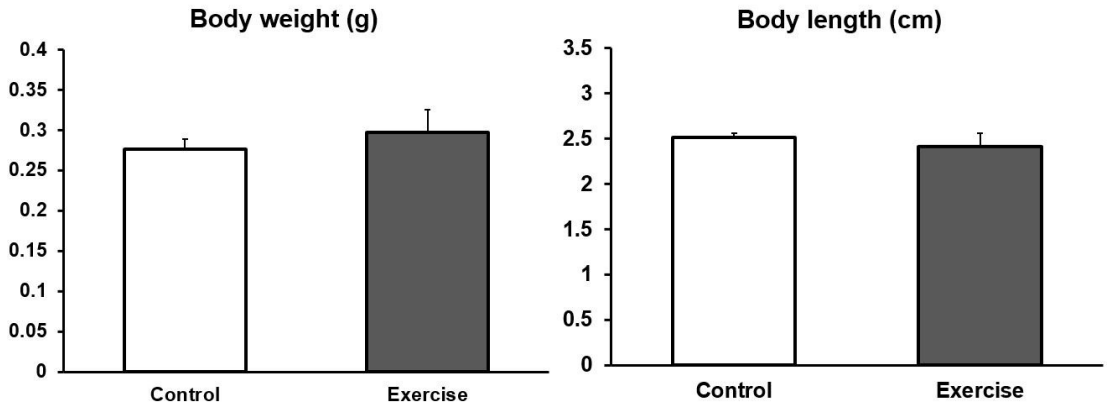

Figure 11: Changes in the macroscopic body weight and size of the zebrafish. The graphs demonstrate average body weight and body length of the exercise and control groups. After physical exercise body weight and body length showed no significant differences between the two groups.

3.2 Bone Histomorphometry

The regions of interest for histomorphometric bone-related parameters within the muscle tissue of fish from the control and the exercise group are illustrated in Figure 12. Morphological analysis of bone formation parameters showed that there was a significant difference regarding several parameters between the exercise group and the control group of adult zebrafish.

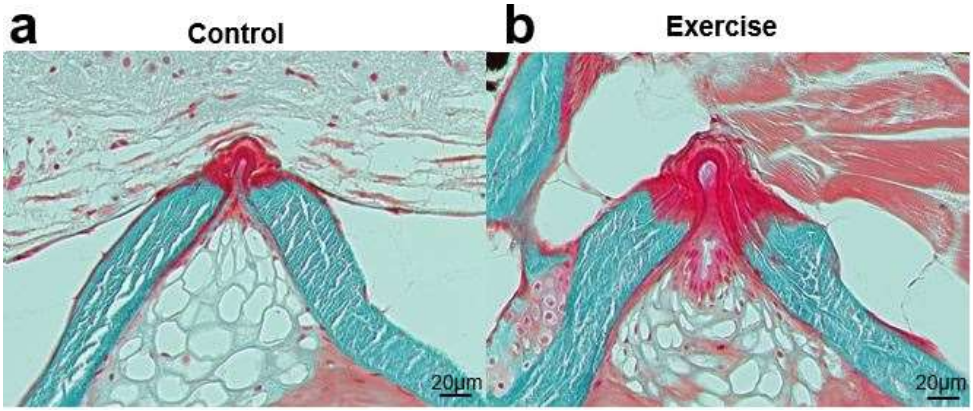

Figure 12: Bone adaptations to swimming exercise in adult zebrafish. The intervertebral region between two adjacent vertebrae is shown on Masson-Goldner trichrome-stained sections in the control group (a) and in the exercise group (b). More osteoblasts and connective tissue were observed in the exercise group compared to control group.

3.2.1 Osteoblast- and osteoid-related bone formation markers

Comparing the control group vs. the exercise group, the number of osteoblasts per bone perimeter (N.Ob/B.Pm) (6.77 ± 0.43 /mm vs. 9.98 ± 0.73 /mm, $p<0.001$) and

the osteoblast surface per bone surface (Ob.S/BS) (3.54 ± 0.30 % vs. 5.47 ± 0.52%, p<0.001) were significantly higher in the exercise group compared to the control group. In the exercise group, the N.Ob/B.Pm (9.93 ± 0.80/mm vs. 10.18 ± 0.51/mm, p=0.53) and the Ob.S/BS (5.57 ± 0.55 % vs. 5.11 ± 0.27%, p=0.07) were compared between the caudal and precaudal area, and there was no significant difference between them (Figure 12,13).

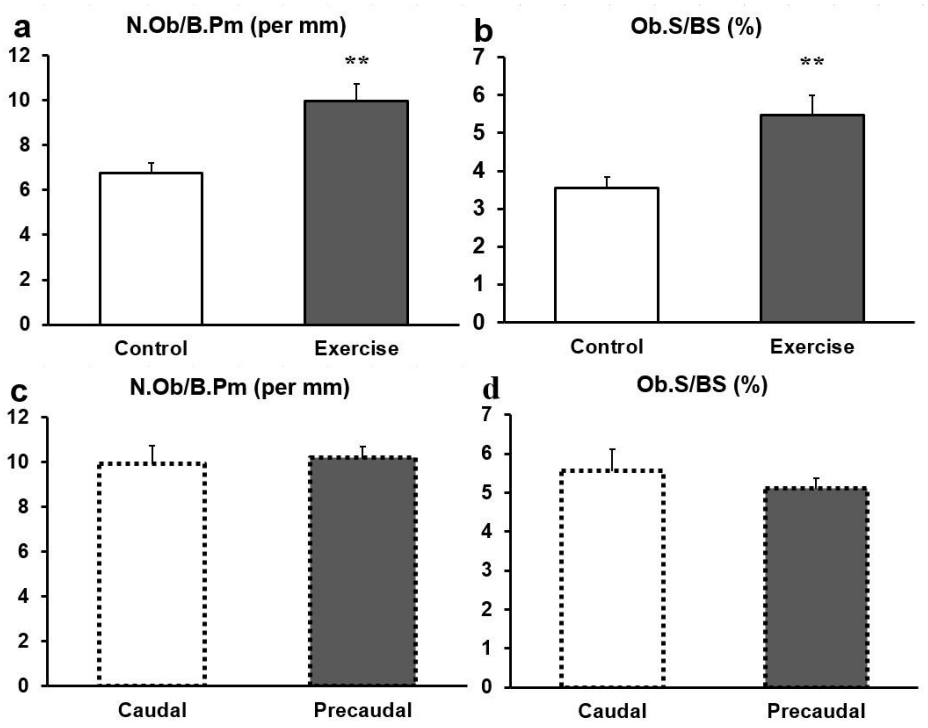

Figure 13: Bone adaptation after swimming exercise in adult zebrafish. (a) Histomorphometryic analysis showed that osteoblast number/bone perimeter (N. Ob/B.Pm) was significantly higher in the exercise group. (b) Osteoblast surface/bone surface (Ob.S/B.S) was significantly higher in the exercise group. (c) (d) Comparing these two parameters between the Caudal vs. the Precaudal area in the exercise group, there were no significant difference. **p < 0.001.

In addition to osteoblastic bone formation parameters, the amount of unmineralized bone matrix (osteoid) yielded differences between the groups. In the control group compared to the exercise group, the osteoid thickness (O.Th) (1.91 ± 0.32 μm vs. 0.83 ± 0.18 μm, p < 0.001) and the osteoid surface per bone surface (OS/BS) (3.04 ± 0.26 % vs. 1.70 ± 0.42 %, p < 0.001) were significantly higher in the control group. In the exercise group the O.Th (0.82 ± 0.18 μm vs. 0.87 ± 0.17 μm, p=0.69) and the OS/BS (1.69 ± 0.41% vs. 1.71 ± 0.46%, p = 0.96) were compared between the caudal and precaudal area and there was no significant difference between them. (Figure 14).

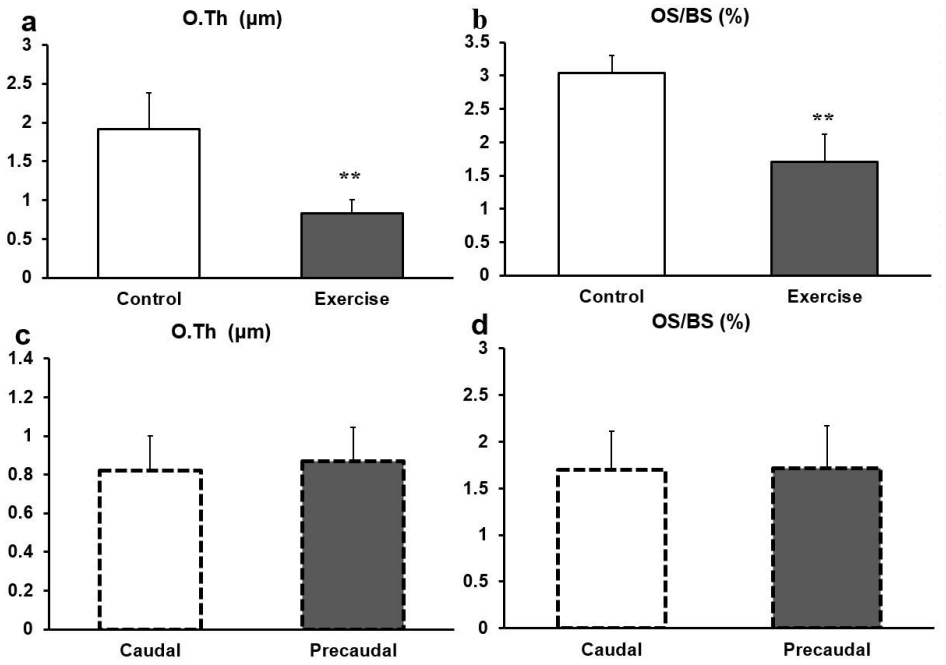

Figure 14: Bone adaptations to swimming exercise in adult zebrafish. The osteoid thickness (O.Th) (a) and the osteoid surface per bone surface (OS/BS) (b) were significantly lower in the exercise group. (c) (d) Comparing these two parameters between the Caudal vs. the Precaudal area in the exercise group, there were no significant difference. **$p < 0.001$.

3.2.2 Tissue and osteocyte characteristics

Comparing the control group and the exercise group regarding the bone volume over total volume (BV/TV) (16.8 ± 1.42 vs. 17.50 ± 1.92, p=0.33) and the osteocyte number/bone area (N.Ot/B.Ar) (159.60 ± 26.09 vs. 188.32 ± 37.00, p=0.01) higher values were observed in the exercise group. There was no significant difference in the BV/TV, however, there was significant difference in the N.Ot/B.Ar between the control and the exercise group. (Figure 15).

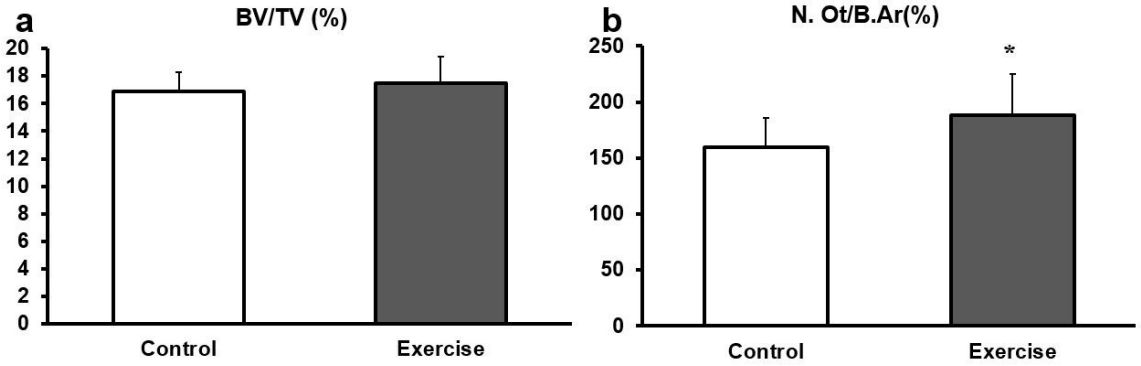

Figure 15: Bone adaptations after swimming exercise in adult zebrafish. There was no significant difference in the bone volume over total volume (BV/TV) (a) and significant difference in the osteocyte number/bone area (N. Ot/B.Ar) (b) between the control and the exercise group. *$p < 0.05$.

3.3 Muscle Histomorphometry

The regions of interest for histomorphometric muscle-related parameters within the muscle tissue of fish from the control and the exercise group is illustrated in Fig. 16. As indicated in Fig. 10, the precaudal vertebral area and the caudal vertebral area (a) (b) were analyzed for muscle histomorphometry. The control group had a lower number of muscle fibers and a lower percentage of fibers containing central nuclei compared to the exercised fish, while in the control group the average size of the muscle fibers was found to be larger compared to the exercise group.

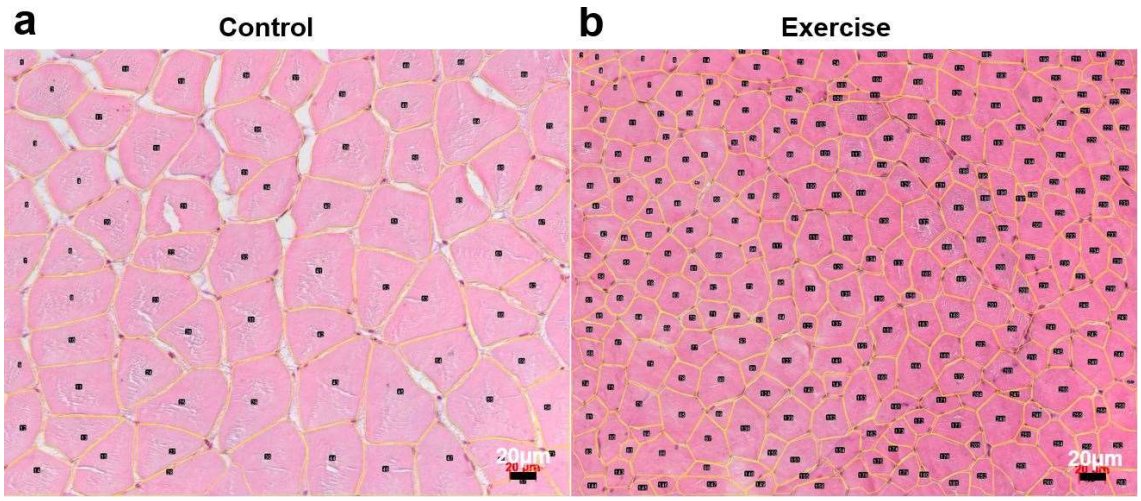

Figure 16: Muscle adaptations to swimming exercise. There was a higher number of muscle fibers in the exercise group. The histological images show the muscle fiber cross sections from control group (a) and exercise group (b) stained by Hematoxylin and Eosin. A higher muscle fiber number per area was identified in the exercise group.

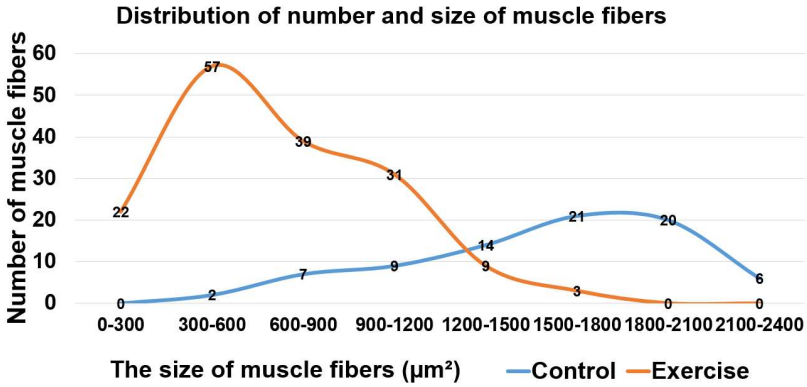

Figure 17: Muscle adaptations to swimming exercise. Data is shown from one caudal section as an example, this diagram shows the distribution of number and size of muscle fibers in both exercise and control group. In the exercise group there was higher number of smaller sized muscle fibers compared to the control group.

3.3.1 Number of muscle fibers

The skeletal muscle adaptation to swimming exercise resulted in a higher number of muscle fibers compared to non-exercised control fish. The control group displayed a significantly lower number of muscle fibers than the exercise group in both precaudal and caudal vertebral areas. The average number of muscle fibers was in the precaudal vertebral area (69.33 ± 5.44 vs. 186.89 ± 23.26, p=0.01), caudal vertebral area (a) (68 ± 4.95 vs. 181.22 ± 19.16, p= 0.009), caudal vertebral area (b) (67.22 ± 4.47 vs. 207.67 ± 23.81, p=0.009) respectively in control group vs. exercise group (Figure 17, 18).

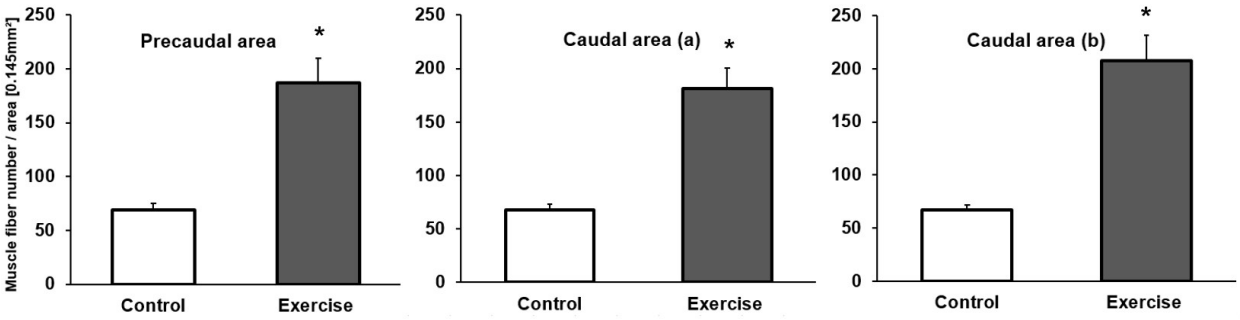

Figure 18: Muscle adaptations to swimming exercise. The graphs show quantification of the number of muscle fibers in the precaudal and the caudal vertebra muscle areas (a) and (b). There were significantly more muscle fibers in the exercise group than the control group in precaudal vertebral area (p=0.03), caudal vertebral area (a) (p= 0.02) and caudal vertebral area (b) (p=0.03). Graphs display the results of the three areas concerning the number of muscle fibers per area, *p < 0.05.

3.3.2 Size of muscle fibers

Analysis showed that in the exercise group the average size of the muscle fibers was significantly smaller compared to the control group in both precaudal and caudal vertebral areas (a) and (b) (Figure 19). The average size of the muscle fibers was for the precaudal vertebral area 1730.60 ± 165.871µm² vs. 670.94 ± 76.21µm², p=0.01, for the caudal vertebral area (a) 1802.70 ± 199.67µm² vs. 722.97 ± 119.19 µm², p=0.003, for the caudal vertebral area (b) 1746.87 ± 84.20µm² vs. 631.62 ± 110.65µm², p=0.002 in control group vs. exercise group, respectively (Figure 19).

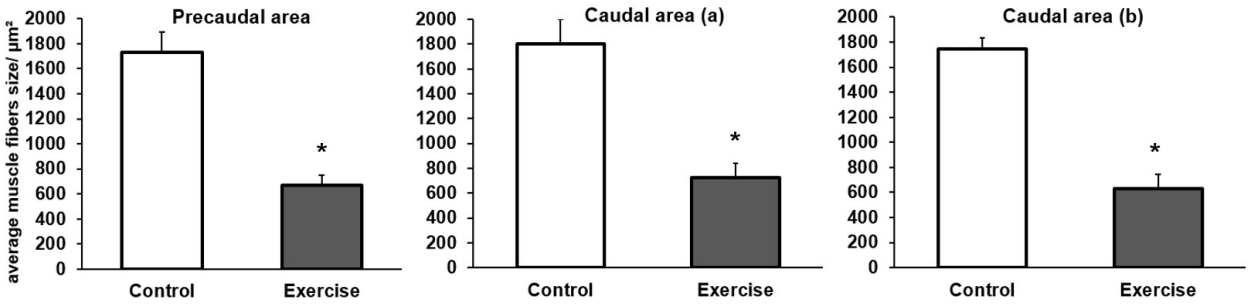

Figure 19: Muscle adaptations to swimming exercise. Physical exercise causes changes in the cross-sectional area of the muscle fibers. The graphs show that in all areas investigated the average size of muscle fibers is significantly smaller in the exercise group compared to control group in all areas; precaudal vertebral area (p=0.01), caudal vertebral area (a) (p=0.003), and caudal vertebral area (b) (p=0.002). *p < 0.05.

3.3.3 Muscle fibers with central nuclei

In the exercise group, more centrally nucleated muscle fibers were identified than in the control group. The average percentage of fibers containing central nuclei was for the precaudal vertebral area 0.44 ± 0.09% vs. 8 ± 0.15% (p < 0.001), for the caudal vertebral area (a) 0.78 ± 0.51% vs. 9.11 ± 1.19% (p=0.006), and for the caudal vertebral area (b) 0.67 ± 0.16% vs. 9.67 ± 1.25% (p=0.004) in control group vs. exercise group, respectively (Figure 20, 21).

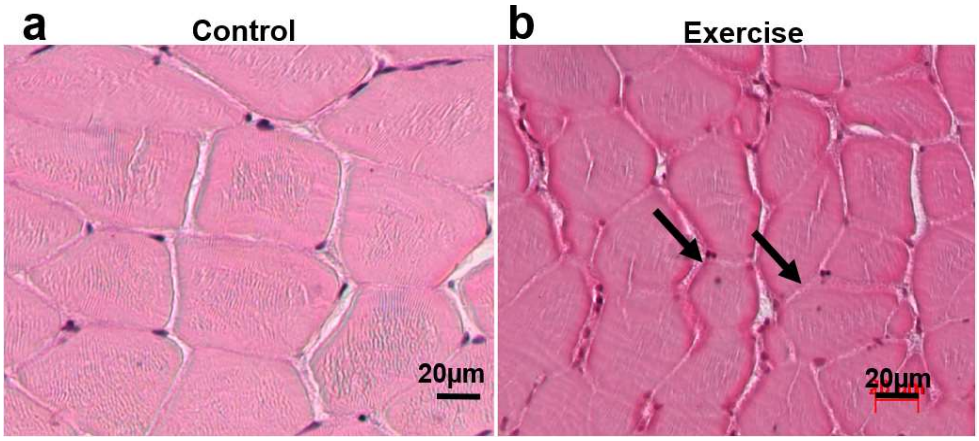

Figure 20: Muscle adaptations to swimming exercise. Physical exercise generated more muscle fibers with central nuclei. The histological image shows the presence of central nucleated muscle fibers in the exercise group (b) and they are generally recognized as regenerated myofibers. Muscle fibers with central nuclei (black arrows) can be observed in the exercise group (b) but rarely in the control group (a).

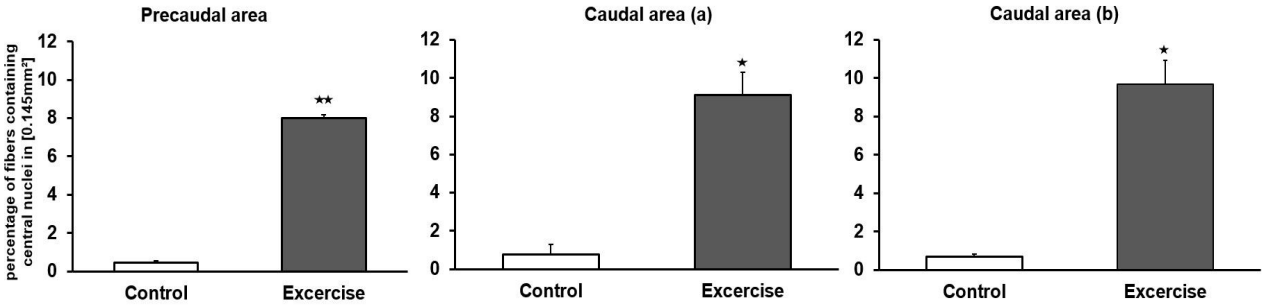

Figure 21: Muscle adaptations to swimming exercise. The graph shows a higher percentage of centrally nucleated muscle fibers in the exercise group compared to the non-exercised group in precaudal vertebral area ($p < 0.001$), caudal vertebral area (a) ($p=0.006$) and caudal vertebral area (b) ($p=0.004$). *$p < 0.05$, **$p < 0.001$.

4 Discussion

Muscle and bone are inextricably linked by genetic, molecular, anatomical and environmental factors, forming a muscle-bone unit. Therefore, it is assumed that increased physical exercise causes corresponding adaptations in both muscle and bone. The aim of the current study was to test the hypothesis that swimming exercise leads to muscle and bone adaptations in adult zebrafish, to quantify the cellular and structural adaptations, and furthermore to compare parameters between the caudal and precaudal areas along the zebrafish spine to test whether musculoskeletal activity leads to region-dependent adaptations.

4.1 Increased physical exercise and musculoskeletal adaptations

This study provides new data on adaptive responses of the musculoskeletal system to increased physical exercise in adult zebrafish. An adequately functioning musculoskeletal system plays an important role in functional capacity, independence, and quality of life. It has been shown that physical exercise positively affects most structural components of the musculoskeletal system which are related to functional capabilities and lowers the risk of degenerative diseases [82]. To better understand the mechanisms behind adaptations to exercise, the zebrafish provides an ideal small-sized animal model. Previous results of a zebrafish study showed that there were significant adaptations in the bone to physical exercise in terms of increased mineralization and bone formation [70], however it has not yet been reported what adaptations in muscle are caused and whether there is difference in some parameters between the caudal and precaudal spine of zebrafish related to the different types of locomotion along the spine.

4.2 Bone adaptations to swimming exercise

One of the most important characteristics of bone lies in its structural and mechanical properties. The adaptive response to increased physical exercise is mediated by osteoblasts which initially secrets new bone tissue as non-mineralized osteoid. Several studies have shown that larger and higher mineral content are found in exercised fishes such as Atlantic salmon and rainbow trout as adaptive response to

load [83] [84]. Data from this study showed a higher number of osteoblasts and related parameters as adaptive response to increased physical exercise, but a smaller degree of vertebral unmineralized osteoid. The vertebra of zebrafish, like vertebrae from other experimental animals and humans, has the ability of adjusting to increased physical exercise [70] [82].

A previous study has shown that sustained swimming reduced the number of fused vertebrae in rainbow trout [85], because an appropriate mechanical load is needed to maintain the integrity of matrix composition and cellularity in the intervertebral area [86]. Moreover, it was established that increasing exercise or load enhances bone development and vertebral bone mineralization (BM) [87], which indicates more unmineralized osteoid becomes mineralized. This is also confirmed by the results of the study at hand, with a low amount of vertebral unmineralized osteoid in response to increased swimming exercise. Bone formation and the mineralization process rely on a crucial interaction between muscle forces and mechanosensitive osteocytes which is derived from the osteoblast. The osteoblast progenitor is recruited to the bone surface where an osteoblast differentiates into a polygonal matrix-producing cells [88]. They connect with existing cells and are embedded in osteoid where they are referred to as osteoid-osteocytes [88]. They become mature osteocytes when the matrix around them mineralizes. Osteocytes are sensitive to mechanical strain in the form of shear stress by muscle forces and translate it into biochemical signals between cells on bone surface for both bone resorption and bone formation [89].

4.3 Muscle adaptations to swimming exercise

It has been shown that increased swimming exercise is associated with muscle growth and muscle growth marker gene expression in adult zebrafish [90], however, it has not yet been clarified what kind of structural, cellular, and molecular changes take place in muscle as a response to increased swimming exercise in adult zebrafish. This current study also tests the hypothesis that physical exercise causes muscle adaptation in adult zebrafish, and aimed to characterize those macroscopic, histological, and cellular adaptations. Wiluam.D et.al [91] has shown that fish which had small muscle fibers increased their muscle mass mainly via increasing in fiber size,

while those animals initially having large fibers responded to training by an increase in fiber number, with less emphasis on change in size of each fiber. The presented results indicate there were no significant differences in body mass between the control and exercise group, however exercised zebrafish showed a higher number of muscle fibers compared to non-exercised control fish and significantly smaller size of the muscle fibers compared to control group, indicating that zebrafish belong to a species with small muscle fibers. However, much of the literature is inconclusive, due to the type of fish, training regimen, exercise period and other fish conditions, which are likely associated with the type of adaptation [91-94]. This study focused on fast-twitch muscle fiber area in zebrafish, since there could be different adaptive responses to increased physical exercise between the fast and slow-twitch muscle fibers.

4.4 Mechanism of muscle adaptations

In small zebrafish, effects of short periods of training during development on aerobic and anaerobic metabolism have been reported [92]. Also in large salmonid fish, effects such as enhanced muscle aerobic potential, increased fiber cross-sectional area, and improved heart performance have been addressed [93] [94]. To discover whether increasing swimming exercise also affects size and number of muscle fibers in adult zebrafish, the number and cross-sectional area of muscle fibers were measured within this study. Result showed that cross-sectional area becomes significantly smaller in response to increased swimming exercise. Exercise-induced muscle fiber injury has been well-described in the literature [95] [96] and it is quite clear that exercise for which a skeletal muscle is not adequately conditioned leads to focal sites of injury distributed within and among the muscle fibers and these adaptations of the muscle reduces the amount of overall injury [97]. Afterwards, muscle injury healing occurs through different phases, such as (a) degeneration and inflammation which is important in the removal of injured tissue and for stimulating regeneration of the damaged fibers, (b) muscle regeneration, and (c) development of fibrosis. In the first few days after injury, muscle degeneration and inflammation occurs, and clear evidence showed that the injured fibers are in the regenerative phase four to five days after injury, and the muscle could completely heal through regeneration [98]. Usually the regeneration process peaks at two weeks and decreases at three to four weeks

after injury [99]. This means muscle undergoes a distinct process such as degeneration, inflammation, regeneration, and fibrosis during the same time. Results of this study therefore indicate that increased swimming exercise lead to continued injury to the muscle fibers in zebrafish skeletal muscle, inducing regeneration of the damaged fibers and growth of new fibers, thus leading to higher numbers of fibers with smaller cross-sections.

4.5 Satellite cells and Myonuclei with muscle adaptations

Satellite cells which are muscle stem cells play an essential role in muscle growth and also in the response to injuries caused by either exercise or disease [64]. Satellite cells are maintained in a quiescent state and upon requirement become activated, proliferating, and fusing with other cells to form or repair myofibers. After injury, satellite cells are activated by specific muscle injury signals and proliferate, produce myogenic precursor cells, known as myoblasts which form new myotubes or fuse with damaged myofibers eventually becoming mature functional skeletal myofibers. This is also why high muscle activity and load often lead to an increase in the transverse size (thickness) of the muscle [100]. Activation of satellite cells is the first step for muscle regeneration. Therefore, satellite cells are considered the most promising target in a cell-based therapy for muscle wasting disorders [101].

Adult muscle fibers are multinuclear and have a symplast structure [100], developed in ontogenesis via fusion of myoblasts. The myonuclei (nuclei of a muscle fiber) which are postmitotic and cannot divide, are located at the periphery of a fiber in the space between myofibrils and sarcolemma. Once the myonuclei migrate from a central position to a subsarcolemmal position, the muscle cells turn into myofibers. An increased number of myonuclei in adult muscle fiber was discovered during a power exercise, experimental working hypertrophy, and post atrophic reloading [102] [103]. The new nuclei in muscle fibers can be caused only through the fusion of satellite cells with the muscle fiber. The satellite cells could offer new nuclei for the muscle fiber during the postnatal period and also for the local regeneration of the injured muscle fibers [104]. Thus, in normal adult muscle the appearance of central nuclei can be an indication of muscle regeneration [105]. Results of this study showed higher percentage of fibers containing central nuclei within the exercise group compared to

control group, indicating that more muscle fibers are generated as skeletal muscle adaptation to increased swimming exercise in zebrafish.

5 Summary-Zusammenfassung

5.1 Summary

A functional musculoskeletal system plays an important role in functional capacity, independence, and quality of life. Physical exercise positively affects most structural components of the musculoskeletal system which are related to functional capabilities and the risk of degenerative disease. This study showed that swimming exercise leads to muscle and bone adaptations in adult zebrafish and quantified the cellular and structural adaptations in terms of increased bone growth and increased muscle regeneration. In particular, one month of physical exercise promoted bone adaptations with increased osteoblastic bone formation and increased mineralization. Muscle adaptations in response to exercise included higher numbers of smaller muscle fibers and central nucleated muscle fibers in exercise group, however the size of muscle fiber was much smaller in exercise group. The potential changes in muscle fiber types after one month of physical exercise needs to be further investigated to better understand the role of specific muscle fiber types during adaptation.

This study strongly supports that the zebrafish is an important and valuable vertebrate model organism in scientific research, specifically an important animal model for musculoskeletal diseases. It also provides new information on adaptive responses of the musculoskeletal system to increased physical exercise in adult zebrafish. This study also points out how exercise experiments in adult zebrafish could foster in-depth analysis of aging-related musculoskeletal diseases.

5.2 Zusammenfassung

Ein intakter Bewegungsapparat spielt eine wichtige Rolle für die körperliche Funktionsfähigkeit, Unabhängigkeit und Lebensqualität. Körperliche Betätigung wirkt sich positiv auf die meisten strukturellen Komponenten des Bewegungsapparates aus, die mit funktionellen Fähigkeiten und dem Risiko von degenerativen Erkrankungen zusammenhängen. Diese Studie zeigte, dass Schwimmtraining bei erwachsenen Zebrafischen zu Muskel- und Knochenanpassungen führt, wie beispielsweise eines erhöhten Knochenaufbaus und einer gesteigerten Muskelregeneration. Das vierwöchige Schwimmtraining induzierte die Anpassung von Knochen in Form von verstärkter Knochenbildung durch erhöhte Osteoblastenaktivität, sowie einer Erhöhung der Mineralisierung von Knochen. Muskelanpassungen als Reaktion auf körperliche Betätigung umfassten eine höhere Anzahl von Muskelfasern sowie von Muskelfasern mit zentral lokalisierten Zellkernen in der Übungsgruppe, jedoch war die Größe der Muskelfasern in der Übungsgruppe signifikant kleiner. Die potenziellen Veränderungen der Muskelfasertypen nach einem Monat körperlicher Betätigung müssen weiter untersucht werden, um die Rolle spezifischer Muskelfasertypen während der Anpassung besser verstehen zu können.

Diese Studie zeigt deutlich, dass der Zebrafisch ein wichtiger Wirbeltiere-Modellorganismus für die biomedizinische Forschung ist, besonders zur Erforschung von Erkrankungen des Bewegungsapparates und des muskuloskelettalen Systems. Die Studie enthält außerdem neue Informationen über die Adaptionen des Bewegungsapparates von erwachsenen Zebrafischen auf erhöhte körperliche Betätigung. Diese Studie weist somit darauf hin, dass die Durchführung von Bewegungsexperimenten am Zebrafisch-Modell eine ausführliche Analyse von alterungs-assoziierten Gewebsveränderungen oder pathologischer Veränderungen ermöglicht.

6 Abbreviations

N.Ob/B.Pm	Number of osteoblasts per bone perimeter
Ob.s/BS	Osteoblast surface per bone surface
N.Ob	Osteoblasts number
O.Th	Osteoid thickness
OS/BS	Osteoid surface per bone surface
BV/TV	Bone Volume over Total Volume
N.Ot/B.Ar	Osteocyte number/bone area
MMA	Methyl methacrylate
DMP	2,4,6-Tris(dimethylaminomethyl)phenol
H&E stain	Hematoxylin and eosin staining
CNFs	Centrally nucleated fibers
BM	Bone mineralization

7 References

[1] Seeman E, Hopper JL, Young NR, Formica C, Goss P, Tsalamandris C (1996). Do genetic factors explain associations between muscle strength, lean mass, and bone density? A twin study. Am. J. Physiol. 270 (2 Pt 1):E320-327.

[2] Frost HM (1997). On our age-related bone loss: insights from a new paradigm. J. Bone Miner. Res. 12(10):1539-1546.

[3] Frost HM, Schonau E (2000). The “muscle–bone” unit in children and adolescents: a 2000 overview. J. Pediatr. Endocrinol. Metab. 13(6):571-590.

[4] Schiessl H, Frost HM, and Jee WS (1998). Estrogen and bone-muscle strength and mass relationships. Bone. 22:1-6.

[5] Turner CH (2000). Muscle-bone interactions, revisited. Bone. 27, pp.339-340.

[6] Fricke O, Schoenau E (2007). The 'Functional Muscle-Bone Unit': probing the relevance of mechanical signals for bone development in children and adolescents. Growth Horm IGF Res. 17(1):1-9.

[7] Schoenau E, Frost HM (2002). The ‘muscle-bone unit’ in children and adolescents. Calcif. Tissue Int. 70(5):405-407.

[8] Schoenau E, Neu CM, Beck B, Manz F, Rauch F (2002). Bone mineral content per muscle cross-sectional area as an index of the functional muscle-bone unit. J. Bone Miner. Res. 17(6):1095-1101.

[9] Rüth EM, Weber LT, Schoenau E, Wunsch R, Seibel MJ, Feneberg R, Mehls O, Tönshoff B. (2004). Analysis of the functional muscle-bone unit of the forearm in pediatric renal transplant recipients. Kidney Int. 66(4):1694-1706.

[10] Turner CH (2000). Muscle–bone interactions, revisited. Bone. 27(3):339-340.

[11] Daly RM , Saxon L , Turner CH , Robling AG , Bass SL (2004). The relationship between muscle size and bone geometry during growth and in response to exercise. Bone. 34(2):281-287.

[12] Liedert A, Kaspar D, Augat P, Ignatius A, Claes L (2005). Mechanobiology of Bone Tissue and Bone Cells. Mechanosensitivity in Cells and Tissues. Moscow: Academia, Part III.

[13] Ami R. A, Cato T. L, Syam P. N (2012). Bone Tissue Engineering: Recent Advances and Challenges. Crit Rev Biomed Eng. 40(5): 363-408.

[14] Rho JY, Kuhn-Spearing L, Zioupos P (1998). Mechanical properties and the hierarchical structure of bone. Med Eng Phys. 20(2):92-102.

[15] Qiu S, Rao DS, Palnitkar S, Parfitt AM (2006). Differences in osteocyte and lacunar density between Black and White American women. Bone. 38: 130-135.

[16] Bonewald L. F (2007). Osteocytes as dynamic multifunctional cells. Ann. N Y Acad. Sci. 1116: 281-290.

[17] You LD, Weinbaum S, Cowin SC, Schaffler MB (2004). Ultrastructure of the osteocyte process and its pericellular matrix. Anat Rec A Discov Mol Cell Evol Biol. 278(2):505-513.

[18] Sauren YM, Mieremet RH, Groot CG, Scherft JP (1992). An electron microscopic study on the presence of proteoglycans in the mineralized matrix of rat and human compact lamellar bone. Anat Rec. 232 (1): 36-44.

[19] Milovanovic P, Zimmermann EA, Riedel C, vom Scheidt A, Herzog L, Krause M, Djonic D, Djuric M, Püschel K, Amling M, Ritchie RO, Busse B (2015). Multi-level

characterization of human femoral cortices and their underlying osteocyte network reveal trends in quality of young, aged, osteoporotic and antiresorptive-treated bone. Biomaterials. 45:46-55.

[20] Hiramatsu K, Asaba Y, Takeshita S, Nimura Y, Tatsumi S, Katagiri N, Niida S, Nakajima T, Tanaka S, Ito M, Karsenty G, Ikeda K (2007). Overexpression of gamma-glutamyltransferase in transgenic mice accelerates bone resorption and causes osteoporosis. Endocrinology. 148(6):2708-2715.

[21] Lorenz-Depiereux B, Bastepe M, Benet-Pagès A, Amyere M, Wagenstaller J, Müller-Barth U, Badenhoop K, Kaiser SM, Rittmaster RS, Shlossberg AH, Olivares JL, Loris C, Ramos FJ, Glorieux F, Vikkula M, Jüppner H, Strom TM (2006). DMP1 mutations in autosomal recessive hypophosphatemia implicate a bone matrix protein in the regulation of phosphate homeostasis. Nat Genet. 38(11):1248-1250.

[22] Marenzana M, Shipley AM, Squitiero P, Kunkel JG, Rubinacci A (2005). Bone as an ion exchange organ: evidence for instantaneous cell-dependent calcium efflux from bone not due to resorption. Bone. 37(4):545-554.

[23] Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K (2007). Targeted Ablation of Osteocytes Induces Osteoporosis with Defective Mechanotransduction. Cell Metabolism. 5(6):464-475.

[24] Mescher A (2015). Junqueira's Basic Histology: Text and Atlas. McGraw-Hill Education Ltd. 14th Edition, Chapter 8. Bone.

[25] Collection of histological, general histology - cartilage and bone. Institute of Histology and Embryology of Charles University in Prague, LF in Pilsen. [Online in Internet.] URL: http://www.lfp.cuni.cz/Histologie/Preparaty/preparaty2E.php?SYSTEM=GeneralHistol_Cartilage&PREPARAT=9. [Stand: 28.09.2018, 10:04].

[26] Shav D, Einav S (2010). The effect of mechanical loads in the differentiation of precursor cells into mature cells. Ann N Y Acad Sci. 1188:25-31.

[27] Buchanan CI, Marsh RL (2002). Effects of exercise on the biomechanical, biochemical and structural properties of tendons. Comp Biochem Physiol A Mol Integr Physiol. 133(4):1101-1107.

[28] Hutson MS, Tokutake Y, Chang MS, Bloor JW, Venakides S, Kiehart DP, and Edwards GS (2003). Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. Science. 300(5616):145-149.

[29] Cowin SC (2001). Bone Mechanics Handbook. CRC Press. Boca Raton, London, New York, Washington, D.C. III, Mechanical and Architectural Properties of Bone.10-1.

[30] Robling AG1, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE, Turner CH (2008). Mechanical Stimulation of Bone in Vivo Reduces Osteocyte Expression of Sost/Sclerostin. J Biol Chem. 283(9):5866-5875.

[31] Pajevic PD (2009). Regulation of Bone Resorption and Mineral Homeostasis by Osteocytes. IBMS BoneKEy. 6(2):63-70.

[32] Manolagas SC (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev. 21(2):115-137.

[33] Rowe P, Sharma S. (2018). Physiology, Bone Remodeling. Treasure Island (FL): StatPearls Publishing.

[34] van der Meulen T, Schipper H, van den Boogaart JG, Huising MO, Kranenbarg S, van Leeuwen JL (2006). Endurance exercise differentially stimulates heart and axial muscle development in zebrafish (Danio rerio). Am J Physiol Regul Integr Comp Physiol. Oct; 291(4):R1040-1048.

[35] Khosla S, Riggs BL (2005). Pathophysiology of age-related bone loss and osteoporosis. Endocrinol Metab Clin North Am. 34(4):1015-1030, xi.

[36] Meakin LB, Galea GL, Sugiyama T, Lanyon LE, Price JS (2014). Age-related impairment of bones' adaptive response to loading in mice is associated with sex-related deficiencies in osteoblasts but no change in osteocytes. J Bone Miner Res. 29(8):1859-1871.

[37] Schwab P, Scalapino K (2011). Exercise for bone health: rationale and prescription. Curr Opin Rheumatol. 23(2):137-141.

[38] Dubińska-Magiera M, Daczewska M, Lewicka A, Migocka-Patrzałek M, Niedbalska-Tarnowska J, Jagla K (2016). Zebrafish: A Model for the Study of Toxicants Affecting Muscle Development and Function. Int J Mol Sci. 17(11): 1941.

[39] Fronter RW, Ochala J (2015). Skeletal Muscle: A Brief Review of Structure and Function. Calcif Tissue Int. 96(3):183-195.

[40] Lindskog C, Linné J, Fagerberg L, Hallström BM, Sundberg CJ, Lindholm M, Huss M, Kampf C, Choi H, Liem DA, Ping P, Väremo L, Mardinoglu A, Nielsen J, Larsson E, Pontén F, Uhlén M (2015). The human cardiac and skeletal muscle proteomes defined by transcriptomics and antibody-based profiling. BMC Genomics. 16(1): 475.

[41] Schiaffino S, Reggiani C (2011). Fiber types in mammalian skeletal muscles. Physiol Rev. 91(4):1447-1531.

[42] Bassel-Duby R, Olson EN (2006). Signaling pathways in skeletal muscle remodeling. Annu Rev Biochem. 75:19-37.

[43] Pette D, Staron RS (2000). Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech. 50(6):500-509.

[44] Hernandez LP, Patterson SE, Devoto SH (2005). The development of muscle fiber type identity in zebrafish cranial muscles. Anat Embryol (Berl). 209(4):323-334.

[45] Luna VM, Daikoku E, Ono F (2015). "Slow" skeletal muscles across vertebrate species. Cell Biosci. 5:62.

[46] van Raamsdonk W, Tekronnie G, Pool CW, van de Laarse W (1980). An immune histochemical and enzymic characterization of the muscle fibres in myotomal muscle of the teleost Brachydanio rerio, Hamilton-Buchanan. Acta Histochem. 67(2):200-216.

[47] Guth L, Samaha FJ (1969). Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. Exp Neurol. 25(1):138-152.

[48] von Hofsten J, Elworthy S, Gilchrist MJ, Smith JC, Wardle FC, Ingham PW (2008). Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo. EMBO Rep. 9(7):683-689.

[49] Blue Histology - Muscle: Skeletal Muscle. School of Anatomy and Human Biology - The University of Western Australia. [Online im Internet.] URL: http://www.lab.anhb.uwa.edu.au/mb140/corepages/muscle/muscle.htm#SKELETAL. [Stand: 28.09.2018, 10:04].

[50] Pette D, Staron RS (2000). Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech, 50:500-509.

[51] Talbot J, Maves L (2016). Skeletal muscle fiber type: using insights from muscle developmental biology to dissect targets for susceptibility and resistance to muscle disease. Wiley Interdiscip Rev Dev Biol. 5(4): 518-534.

[52] Javan R, Horvath JJ, Case LE, Austin S, Corderi J, Dubrovsky A, Kishnani PS, Bashir MR (2013). Generating color-coded anatomic muscle maps for correlation of quantitative magnetic resonance imaging analysis with clinical examination in neuromuscular disorders. Muscle Nerve 48:293-295.

[53] Fortin M, Videman T, Gibbons LE, Battié MC (2014). Paraspinal muscle morphology and composition: a 15-yr longitudinal magnetic resonance imaging study. Med Sci Sports Exerc. 46:893-901.

[54] Booth FW, Roberts CK (2008). Linking performance and chronic disease risk: indices of physical performance are surrogates for health. Br J Sports Med. 42(12):950-952.

[55] Leblanc PJ, Howarth KR, Gibala MJ (2004). GJ.Heigenhauser. Effects of 7 wk of endurance training on human skeletal muscle metabolism during submaximal exercise. J Appl Physiol (1985). 97(6):2148-2153.

[56] Endo T (2015). Molecular mechanisms of skeletal muscle development, regeneration, and osteogenic conversion. Bone. 80:2-13.

[57] Qaisar R, Bhaskaran S, Van Remmen H. (2016). Muscle fiber type diversification during exercise and regeneration. Free Radic Biol Med. 98:56-67.

[58] Magaudda L, Di Mauro D, Trimarchi F, Anastasi G (2004). Effects of physical exercise on skeletal muscle fiber: ultrastructural and molecular aspects. Basic Applied Myology. 14: 17-21.

[59] Charge SB, Rudnicki MA (2004). Cellular and molecular regulation of muscle regeneration. Physiological Reviews. 84:209-238.

[60] Pearson AM (1990). Muscle growth and exercise. Crit Rev Food Sci Nutr. 29 (3):167-196.

[61] Seene T, Kaasik P (2013). Muscle damage and regeneration: Response to exercise training. Health. 5-6B: 136-145.

[62] Folland JP, Williams AG (2007). The adaptations to strength training: morphological and neurological contributions to increased strength. Sports Medicine. 37(2):145-68.

[63] Crameri RM, Langberg H, Magnusson P , Jensen CH , Schrøder HD, Olesen JL, Suetta C, Teisner B, Kjaer M (2004). Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. J. Physiol. 558(Pt 1): 333-340.

[64] C. O'Reilly, B. McKay, S. Phillips, M. Tarnopolsky, G. Parise (2008). Hepatocyte growth factor (HGF) and the satellite cell response following muscle lengthening contractions in humans. Muscle Nerve. 38(5):1434-1442.

[65] Mackey AL, Holm L, Reitelseder S, Pedersen TG, Doessing S, Kadi F, Kjaer M (2011). Myogenic response of human skeletal muscle to 12 weeks of resistance training at light loading intensity. Scand. J. Med. Sci. Sports. 21(6):773-782.

[66] Petrella JK, Kim JS, Mayhew DL, Cross JM, Bamman MM (2008). Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. J Appl Physiol (1985).104(6):1736-1742.

[67] Roth SM, Martel GF, Ivey FM, Lemmer JT, Tracy BL, Metter EJ, Hurley BF, Rogers MA (2001). Skeletal muscle satellite cell characteristics in young and older men

and women after heavy resistance strength training. J Gerontol A Biol Sci Med Sci. 56(6):B240-247.

[68] Thornell LE, Lindström M, Renault V, Mouly V, Butler-Browne GS (2003). Satellite cells and training in the elderly. Scand J Med Sci Sports. 13(1):48-55.

[69] Jarolim EJ (2013). Comparative Medicine: Anatomy and Physiology. Springer.4: Locomotor principles: Anatomy and Physiology of Skeletal Muscles: 45-58.

[70] Suniaga S, Rolvien T, vom Scheidt A, Fiedler I A. K, Bale HA, Huysseune A, Eckhard Witten P, Amling M, Busse, B (2018). Increased mechanical loading through controlled swimming exercise induces bone formation and mineralization in adult zebrafish. Sci Rep. 26; 8(1):3646.

[71] Zarins ZA, Wallis GA, Faghihnia N, Johnson ML, Fattor JA, Horning MA, Brooks GA (2009). Effects of Endurance Training on Cardiorespiratory Fitness and Substrate Partitioning in Postmenopausal Women. Metabolism. 58(9):1338-1346.

[72] Shi X, Garry DJ (2006). Muscle stem cells in development, regeneration, and disease. Genes Dev. 20(13):1692-1708.

[73] Narita S, Yorifuji H (1999). Centrally nucleated fibers (CNFs) compensate the fragility of myofibers in mdx mouse. Neuroreport. 19; 10(15):3233-3235.

[74] Wang C, Yue F, Kuang S (2017). Muscle Histology Characterization Using H&E Staining and Muscle Fiber Type Classification Using Immunofluorescence Staining. Bio Protoc. 20: 7(10).

[75] Patton EE, Zon LI (2001). The art and design of genetic screens: Zebrafish. Nature Rev Genet. 2: 956-966.

[76] Briggs JP (2002). The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol. 282: R3-9.

[77] Gerhard GS (2003). Comparative aspects of zebrafish (Danio rerio) as a modelforaging research. Exp Gerontol. 38(11-12):1333-1341.

[78] Kari G, Rodeck U, Dicker AP (2007). Zebrafish: An Emerging Model System forHuman Disease and Drug Discovery. Clin Pharmacol Ther. 82(1):70-80.

[79] Lieschke GJ, Currie PD (2007). Animal models of human disease: zebrafish swiminto view. Nature Rev Genet. 8: 353-367.

[80] Reed B, Jennings M (2010). Guidance on the housing and care of Zebrafish, Danio rerio. 4. Housing and care: 21-41. [Online im Internet.] URL: https://www.aaalac.org/accreditation/RefResources/ZebrafishReference.pdf. [Stand: 28.09.2018, 10:04].

[81] Villamizar N, Vera LM, Foulkes NS, Sánchez-Vázquez FJ (2014). Zebrafish. 11(2):173-81.

[82] Totland GK, Fjelldal PG, Kryvi H, Løkka G, Wargelius A, Sagstad A, Hansen T, and Grotmol S (2011). Sustained swimming increases the mineral content and osteocyte density of salmon vertebral bone. J. Anat. 219(4):490-501.

[83] Ytteborg E, Torgersen JS, Pedersen ME, Helland SJ, Grisdale-Helland B, and Takle H (2013). Exercise induced mechano-sensing and Substance P mediated bone modeling in Atlantic salmon. Bone. 53(1):259-268.

[84] Deschamps MH, Girondot M, Labbé L, Sire JY (2009). Changes in vertebral structure during growth of reared rainbow trout, Oncorhynchus mykiss (Walbaum): a new approach using modelling of vertebral bone profiles. J Fish Dis. 32(3):233-246.

[85] Deschamps MH, Labbé L, Baloche S, Fouchereau-Péron M, Dufour S, Sire J Y (2009). Sustained exercise improves vertebral histomorphometry and modulates hormonal levels in rainbow trout. Aquaculture. 296:3-4:337-346.

[86] Walsh AL, Lotz JC (2004). Biological response of intervertebral disc to dynamic loading. J. Biomech. 37(3):329-337.

[87] Turner CH (1998). Three rules for bone adaptation to mechanical stimuli. Bone. 23(5):399-407.

[88] Bonewald LF (2007). Osteocytes as dynamic multifunctional cells. Ann. N. Y. Acad. Sci. 1116:281-290.

[89] Palumbo C (1986). A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos. Cell Tissue Res. 246(1):125-131.

[90] Palstra AP, Tudorache C, Rovira M, Brittijn SA, Burgerhout E, van den Thillart GE, Spaink HP, Planas JV (2010). Establishing zebrafish as a novel exercise model: Swimming economy, swimming-enhanced growth and muscle growth marker gene expression. PLoS One. 5(12).

[91] Davison, W (1989). Training and its effects on teleost fish. Comp. Biochem. Physiol. 94A:1-10.

[92] Bagatto B, Pelster B, and Burggren WW (2001). Growth and metabolism of larval zebrafish: effects of swim training. J Exp Biol. 204(Pt 24):4335-4343.

[93] Davison W, Goldspink G (1978). The effect of training on the swimming muscles of the goldfish (Carassius auratus). J Exp Biol. 74:115-122.

[94] Davison W (1997). The effects of exercise training on teleost fish, a review of recent literature. Comp Biochem Physiol A. 117: 67-75.

[95] Armstrong RB (1990). Initial events in exercise-induced muscular injury. Med Sci Sports Exerc. 22(4):429-435.

[96] Stauber WT (1989). Eccentric action of muscles: physiology, injury, and adaptations. Exerc Sport Sci Rev. 17:157-85.

[97] Armstrong RB, Warren GL, Warren JA (1991). Mechanisms of Exercise-Induced Muscle Fibre Injury. Sports Med. 12(3):184-207.

[98] Armstrong RB, Ogilvie RW, Schwane JA (1983b). Eccentric exercise-induced injury to rat skeletal muscle. J Appl Physiol Respir Environ Exerc Physiol. 54(1):80-93.

[99] Chargé SB, Rudnicki MA (2004). Cellular and molecular regulation of muscle regeneration. Physiol Rev. 84(1):209-238.

[100] Shenkman BS, Turtikova OV, Nemirovskaya TL, Grigoriev AI (2010). Skeletal Muscle Activity and the Fate of Myonuclei. Acta Naturae. 2(2):59-66.

[101] Berardi E, Annibali D, Cassano M, Crippa S, Sampaolesi M (2014). Molecular and cell-based therapies for muscle degenerations: a road under construction. Front Physiol. 5:119.

[102] Oishi Y, Ogata T, Yamamoto KI, Terada M, Ohira T, Ohira Y, Taniguchi K, Roy RR (2008). Cellular adaptations in soleus muscle during recovery after hindlimb unloading. Acta Physiol (Oxf).192(3):381-395.

[103] Mitchell PO, Pavlath GK (2001). A muscle precursor cell-dependent pathway contributes to muscle growth after atrophy. Am J Physiol Cell Physiol. 281(5): C1706-1715.

[104] Grounds MD (1991). Towards understanding skeletal muscle regeneration. Pathol Res Pract. 187(1):1-22.

[105] Huard J, Li Y, Fu FH (2002). Muscle injuries and repair: current trends in research. J Bone Joint Surg Am. 84-A (5):822-832.

8 Appendix

Hematoxylin and eosin staining (H&E stain)

Universitätsklinikum Hamburg-Eppendorf

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30.3.2015

2. Verfahren und Leistung 2.02 Histolabor	**2.02.5 HE - Färbung**	**2.02.5** Anlage 03 Version 03

Paraffinschnitte bei 60° im Wärmeschrank 30-45 Min. fixieren

-	2x Xylol	je 5 Min. entparaffinieren
-	2x Ethanol abs.	je 2 Min.
-	Ethanol 96%	2 Min.
-	Ethanol 80%	2 Min.
-	Ethanol 70%	2 Min.
-	Ethanol 50%	2 Min.
-	Aqua dest.	spülen(3-5x eintauchen)
-	Mayers Hämalaunlsg.	10 Min.
-	Leitungswasser	2 Min.
-	HCL-Alkohol	2-3x eintauchen
-	Leitungswasser	10 Min.bläuen(fließend wässern oder Wasser auswechseln)
-	Eosinlsg. 0,1%	2 Min.
-	Ethanol 80%	kurz spülen
-	Ethanol 96%	2 Min.
-	2x Ethanol abs.	je 2 Min.
-	3x Xylol	je 5 Min.
-	Eindecken	Eukitt/DXP

Färbeergebnis:

blau-violett:	Zellkerne, Bakterien,Kalk
rot:	Bindegewebe,Protein, Keratin,Cytoplasma, Interzellularsubstanzen

Toluidine blue staining

Universitätsklinikum Hamburg-Eppendorf

IOBM Institut für Osteologie und Biomechanik
Institut für Osteologie und Biomechanik
Direktor: Prof. Dr. Michael Amling
08.04.2015

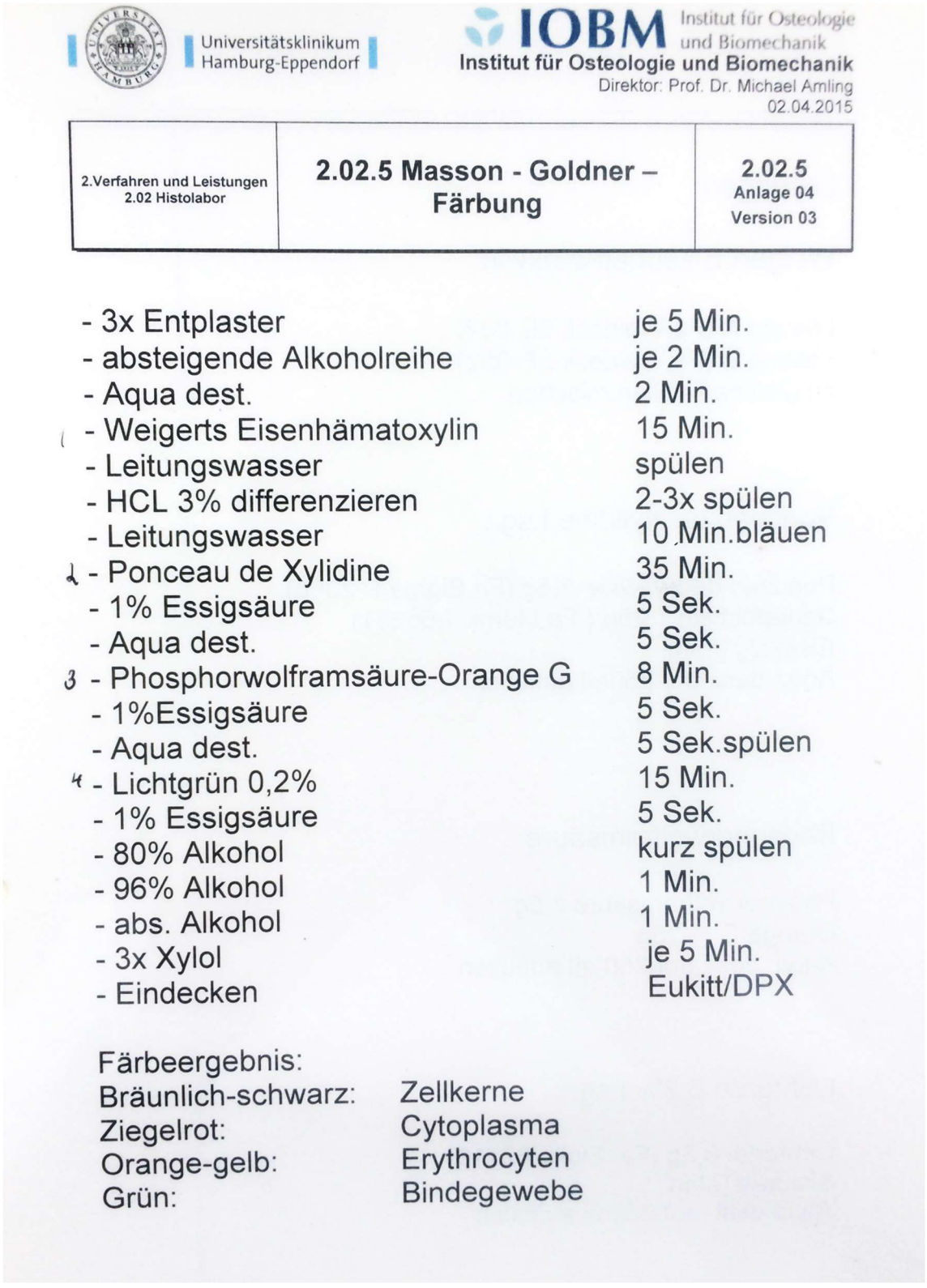
2.Verfahren und Leistungen 2.02 Histolabor	**2.02.5 Toluidin-Färbung**	**2.02.5** Anlage 01 Version 03

- 3x Entplaster	je 5 Min.
- 2x Ethanol abs.	je 2 Min.
- Ethanol 96%	2 Min.
- Ethanol 80%	2 Min.
- Ethanol 70%	2 Min.
- Ethanol 50%	2 Min.
- Aqua dest.	spülen
- Toluidinblau 1%, ph 4,5	30 Min.
- Aqua dest.	spülen
- ~~Ethanol 50%~~	spülen
- ~~Ethanol 70%~~	2 Min.
- Ethanol 80%	~~2 Min~~. kurz spülen
- Ethanol 96%	2 Min.
- 2x Ethanol abs.	je 2 Min.
- Xylol	je 5 Min.
- Eindecken	Eukitt/DPX

Färbeergebnis:

Dunkelblau:	Zellkerne
Blau:	Osteoblasten
Türkisblau:	Osteoklasten
Hellblau:	mineralisierter Knochen (mit dunkleren Kittlinien)
Violett:	Knorpel

Masson-Goldner trichrome staining



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02.04.2015

2.Verfahren und Leistungen 2.02 Histolabor	**2.02.5 Masson - Goldner – Färbung**	**2.02.5** Anlage 04 Version 03

- 3x Entplaster	je 5 Min.
- absteigende Alkoholreihe	je 2 Min.
- Aqua dest.	2 Min.
- Weigerts Eisenhämatoxylin	15 Min.
- Leitungswasser	spülen
- HCL 3% differenzieren	2-3x spülen
- Leitungswasser	10 Min.bläuen
- Ponceau de Xylidine	35 Min.
- 1% Essigsäure	5 Sek.
- Aqua dest.	5 Sek.
- Phosphorwolframsäure-Orange G	8 Min.
- 1%Essigsäure	5 Sek.
- Aqua dest.	5 Sek.spülen
- Lichtgrün 0,2%	15 Min.
- 1% Essigsäure	5 Sek.
- 80% Alkohol	kurz spülen
- 96% Alkohol	1 Min.
- abs. Alkohol	1 Min.
- 3x Xylol	je 5 Min.
- Eindecken	Eukitt/DPX

Färbeergebnis:

Bräunlich-schwarz:	Zellkerne
Ziegelrot:	Cytoplasma
Orange-gelb:	Erythrocyten
Grün:	Bindegewebe

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10 Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

11 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Mamuti Maiwulanjiang
Hamburg, den 29.10.2018