Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

2023

Doctoral thesis

Using of prolonged releasing drug systems in fish reproduction

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Doctoral thesis by
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Title:
Using of prolonged releasing drug systems in fish reproduction
Využívání dlouhodobého uvolňování léčiv v reprodukci ryb

Doctoral study program:
Zootechnics / Fishery, Combined

Knowles, J., 2023. Using of prolonged releasing drug systems in fish reproduction. Doctoral thesis. University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Czech Republic, 122 pp. ISBN ISBN 978-80-7514-189-7 (with the summary in English and Czech)
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. General overview

Teleosts represent an advanced group of vertebrates with approximately 30 000 species covering 96% of the fish class. While teleosts can display most varieties of reproduction described in vertebrates (Helfman et al., 2009), the vast majority are gonochoristic with external fertilization via release of eggs and sperm into water (Patzner, 2008). Environmental factors such as water temperature, photoperiod, and food availability interact with fish biology to impact reproductive success, with hormones playing a crucial role (Abraham et al., 2009). The most important control mechanism of the fish reproductive system is the neuroendocrine hypothalamo-pituitary-gonadal (HPG) axis consisting of the hypothalamus, pituitary, and gonads, which essentially operate as a single unit (Figure 1).

1.1.2. Neuroendocrine regulation of fish reproduction

Gonadotropin releasing hormone

The master regulator of the reproductive cycle in vertebrates is gonadotropin releasing hormone (GnRH), a neurohormone produced by the hypothalamus that controls the anterior pituitary and thus production of the gonadotropins (GTH) such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Weltzien et al., 2004). Formerly, GnRH was called luteinizing hormone–releasing hormone based on its LH stimulatory activity, but was later renamed to GnRH following the findings of its FSH release activity (Okamura et al., 2013).

Teleosts express multiple forms of GnRH and GnRH receptors (GnRHr) (Tostivint, 2011). An increasing number of GnRH isoforms are identified in vertebrates, and at least two forms are found in brain of all vertebrate species. While GnRH variants have traditionally been named for the species in which they were first described, in response to the increasing number of identified GnRH subunits, a new classification based on phylogenetic characteristics has been proposed (Kah et al., 2007). Three categories of GnRH are present in teleosts, regardless of the number of isoforms. GnRH1 is present in most vertebrates, with the exception of some fish species. GnRH2 is found in all vertebrates except rodents, and GnRH3 is found only in fishes (Okubo and Nagahama, 2008).

GnRH1 is produced by neurons located in the preoptic area of the hypothalamus that project primarily into the pituitary, where they regulate reproduction via GTH release (Amano et al., 2004). GnRH1 also controls the production of growth hormone, prolactin, and somatolactin. It is found in all terrestrial vertebrates and some teleosts in species-specific forms such as mammalian GnRH (mGnRH), sea bream GnRH (sbGnRH), pejerrey GnRH (pjGnRH), catfish GnRH (catGnRH), herring GnRH (hGnRH), and whitefish GnRH (wfGnRH) (Kah et al., 2007). GnRH2 includes a single peptide that was first identified in chicken (cGnRH-II) and is found in most tetrapods and teleosts (Oka, 2010). Neurons expressing cGnRH-II are concentrated mainly in the in the midbrain tegmentum and project throughout the brain (Steven et al., 2003). The GnRH3 form is specific to teleost fishes. Its neurons are located in the olfactory bulb but project into the entire brain (Wirsig-Wiechmann and Oka, 2002). GnRH3 participates the control of reproductive behaviour such as nesting, competition, and spawning (Ogawa et al., 2006). It is the key reproductive hormone in cyprinids and salmonids (Umatani and Oka, 2019).

The activity of GnRH is mediated by specific GnRHr that are members of the G-protein coupled receptors. They are generally localized in the brain but have been found in gonads and other peripheral tissue. However, the distribution of GnRHr is limited in teleosts. GnRHr transmits the actions of GnRH though the neural and endocrine system (Lethimonier et al., 2004). In parallel with GnRH, up to five GnRHrs have been discovered in teleosts (Moncaut et al., 2005).

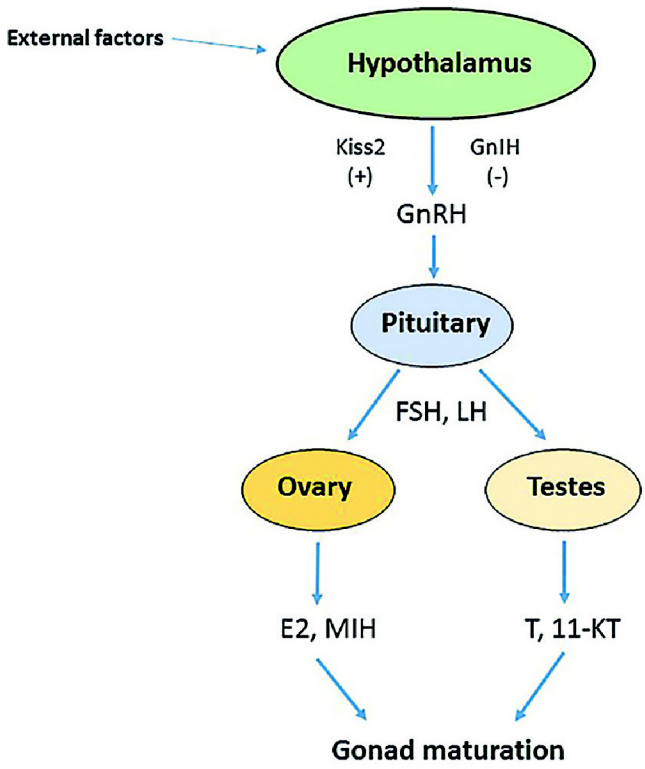

Figure 1. *Hypothalamic-Pituitary-Gonad axis. Gonadotropin releasing hormone (GnRH); follicle-stimulating hormone (FSH); luteinizing hormone (LH); Kisspeptin (Kiss2); Gonadotropin inhibitory hormone (GnIH).*

Kisspeptin

Kisspeptin is a neuropeptide encoded by the KISS1/kiss1 gene (Lee et al., 1996) that regulates the HPG axis (Kitahashi et al., 2009) and the initiation of reproductive maturation (Kitahashi et al., 2009; Shahjahan et al., 2010). Kisspeptin is expressed in brain, primarily in the hypothalamus (Kitahashi et al., 2009), but has been found in intestine, kidney, liver, pancreas, and gonads (Selvaraj et al., 2010). In mammals, kiss1 coding for kisspeptin and kiss1r coding for the receptor have been found (Ohga et al., 2018). In teleosts such as medaka *Oryzias latipes*, zebrafish *Danio rerio*, redfish *Sciaenops ocellatus*, and seabass *Lateolabrax japonicus*, two kisspeptin genes (kiss1 and kiss2) and two kisspeptin receptors (kiss1ra and kiss1rb) have been identified. In species including pufferfish, Senegalese sole *Solea senegalensis*, grass puffer *Takifugu niphobles*, and Atlantic halibut *Hippoglossus hippoglossus* only kiss2 and kiss1rb have been reported (Mechaly et al., 2010; Shahjahan et al., 2010; Shi et al., 2010).

Gonadotropins

In vertebrates, GnRH binds to its receptors and releases GTH, which maintains the function of gonads and plays a key role in the regulation of gametogenesis, steroidogenesis, and sexual behaviour (Yan et al., 2012; Bédécarrats, 2015). Gonadotropins FSH and LH are produced by gonadotropic cells in the pituitary and released into the bloodstream for transfer to the gonads. They comprise heterodimeric glycoproteins with two non-covalently associated subunits, α and β. The gonadotropins have a common subunit, glycoprotein hormone α, that consists of 121 amino acids, and a specific β subunit called FSHβ with 118 amino acids and LHβ with 121 amino acids (Yan et al., 2012; Burow et al., 2019). The GTH binds to luteinizing

hormone receptor (LH-R) and follicle-stimulating hormone receptor (FSH-R), both expressed in gonad (Simoni et al., 1997; Yan et al., 2012).

The gonads release steroid hormones in response to GTH, allowing reproduction and playing a feedback role in control of the pituitary. In teleosts, FSH stimulates follicle growth in the ovary in females and triggers spermatogenesis in males, while LH controls the final process of ovulation and spermiation (Levavi-Sivan et al., 2010). In males, the highest concentrations of LH are found during spermiation and is related to the manifestation of secondary sexual characteristics. In females, the LH level increases during the final stages of oocyte maturation and stimulates the production of dihydroxyprogesterone (17α-20β), which is involved in the haploid processes prior to ovulation (Beshay and Carr, 2017).

The main sex steroids released from the gonads are 17β oestradiol (E2) in females and testosterone (T) and 11-ketotestosterone (11-KT) in males. The GTHs evoke the synthesis of maturation-inducing hormones/steroids (MIH or MIS), including 17α, 20β-dihydroxyprogesterone (DHP) and 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) (Kime, 1993).

Gonadotropin inhibitory hormone

Gonadotropin inhibitory hormone (GnIH) is a hypothalamic dodecapeptide that impedes GTH release through the inhibition of GnRH and kisspeptin, offering multifactorial control of reproduction (Ma et al., 2020). In teleosts, GnIH is primarily expressed in brain and pituitary, but has also been reported in eye, testis, ovary, spleen, kidney, and muscle (Di Yorio et al., 2016; Sawada et al., 2002). Gonadotropin inhibitory hormone acts on the GnRH neurons by binding to G-protein-coupled GnIH receptors (Wang et al., 2015).

Unlike mammals and birds, the physiological function of GnIH in teleosts is not described, and differing effects have been recorded in fish. In mature female Nile tilapia *Oreochromic niloticus*, administration of GnIH increased levels of both LH and FSH (Biran et al., 2014). In goldfish *Carassius auratus*, GnIH can have either stimulatory or inhibitory effects on GTH expression, depending on the reproductive phase. In the early stages of gonad maturation, GnIH inhibits expression of GTH, but it shows a stimulatory effect in the spawning period (Moussavi et al., 2012; Qi et al., 2013). A negative effect of GnIH on GTH levels has been reported in European sea bass *Dicentrarchus labrax* and zebrafish (Paullada-Salmerón et al., 2016; Zhang et al., 2010). These findings indicate that, in fish, the physiological action of GnIH depends on the species, sex, and reproductive phase (Paullada-Salmerón et al., 2016).

1.1.3. Hormonal control of spermatogenesis

In general, the male reproductive system is comprised of the spermatic duct, testicular main duct, and testes (Nagahama, 1983). Both germ and somatic cells are present in testes. The testicular main duct and spermatic duct allow nutrition, steroid expression, and storage of spermatozoa and seminal fluid (Alavi et al., 2008). In teleosts, the testis comprises interstitial and lobular compartments. The interstitial compartment contains Leydig cells, which synthesise androgen sex steroids in response to the activity of gonadotropins (Nagahama, 1983). The lobular compartment contains germ cells and Sertoli cells that are in continuous interaction and present at different developmental stages. Spermatogenesis occurs in testicular cysts created by Sertoli cells in stages that include differentiated spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (Schulz et al., 2010). Spermatogenesis stages include mitotic proliferation of the spermatogonia, the meiosis division of the primary spermatocyte, and the transformation of the haploid

spermatids into flagellated spermatozoa (Nagahama, 1983). Sperm maturation occurs in the spermatic duct (Morisawa and Morisawa, 1986).

External factors including photoperiod and temperature stimulate the hypothalamus to release GnRH, which influences release of GTH from the pituitary. In teleosts, FSH controls the early stages of testicular development and the function of Sertoli cells, while LH controls the subsequent testicular maturation and function of Leydig cells (Schulz et al., 2010). Follicle stimulating hormone acts on Leydig cells to produce 11-KT and thus induces expression of activin B by the Sertoli cells. Activin B stimulates spermatogonia to undergo mitosis, which leads to spermatocyte formation and spermatogenesis (Miura and Miura, 2003). The process is complex and involves, among other hormones, insulin-like growth factors and progestin (17α, 20β-dihydroxy-4-pregnen-3-one), inhibin (Miura and Miura, 2001), and gonadal soma-derived growth factor (Sawatari et al., 2007). Mature spermatozoa are released from testicular lobules into the sperm duct, where sperm maturation occurs under the control of progestin (Miura and Miura, 2003).

Testicular steroids play a pivotal role in teleost spermatogenesis. Steroid regulation of spermatogenesis includes negative and positive responses at different level of the HPG axis (Schulz et al., 2010). As in all vertebrates, 11-KT and T are the major androgenic steroids in spermatogenesis in teleosts. They promote the expression of genes that control spermatogenesis in fish (Le Gac et al., 2008), such as the double-sex- and mab-3-related transcription factor 1, transcription factor AP-1, transcription factor E2F4, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, and transcription factor SOX-8 (Schulz et al., 2010). The level of 11-KT and T increases as spermatogenesis continues and significantly decreases during spermiation (Schulz et al., 2010). The physiological role of E2 in spermatogenesis in teleosts is unclear. However, it is known that low levels of E2 positively affect spermatogenesis while high levels show negative effects (Lahnsteiner et al., 2006; Chaves-Pozo et al., 2007). The final maturation of spermatozoa is controlled by the steroids DHP and 20β-S. In teleosts, DHP induces spermiation and exerts a positive effect on sperm production (Miura and Miura, 2003) and motility (Tubbs and Thomas, 2008).

1.1.4. Hormonal control of oogenesis

Ovaries occur in pairs in majority of teleosts and may differ in physiological organisation and oocyte development (Jamieson, 2009). The primary cell in the ovary is the oogonium. In general, oogonia are transformed into primary oocytes via meiotic division, after which primary oocytes undergo a growth phase and accumulate a reserve of nutrition. The growth period is divided into primary and secondary stages. At the primary stage, mRNA is accumulated, and the first cortical alveoli appear (Murua and Saborido-Rey, 2003). In the secondary stage, vitellogenin derived from the blood is stored in yolk vesicles, usually found in the centre of the oocyte. When the growth phase is complete, the oocyte enters the maturation stage, in which the oocytes are in meiotic arrest and meiosis is re-initiated. Once meiosis division is complete, the egg is release by the rupture of the follicle, completing oogenesis (Lubzens et al., 2010). The phases of oocyte development are shown in Figure 2.

As in spermatogenesis, oogenesis is under control of the HPG axis. The initial stages of oogenesis, follicle growth and vitellogenesis, are controlled by FSH, while the later stages, oocyte maturation and ovulation, are regulated by LH (Levavi-Sivan et al., 2010). In addition to its role in the initial stages of oogenesis, FSH is important in vitellogenesis through its stimulation of E2 synthesis in the ovarian follicles, which controls the synthesis and release of vitellogenin (Yaron and Levavi-Sivan, 2011). Vitellogenesis is a key process in biosynthesis, transport, and absorption of vitellogenin, the main component of the egg yolk protein and

a critical nutritional reserve for the developing embryo. Its synthesis occurs in liver and is controlled by E_2 (Lubzens et al., 2010).

In the growth phase, post-vitellogenic oocytes are in meiotic arrest and may remain in this stage for months (Yaron and Levavi-Sivan, 2011) until the LH level increases and the oocytes undergo the maturation phase. Luteinizing hormone acts via LH receptors in the granulosa cells and stimulates the synthesis of maturation-inducing steroids (MIS) (Nagahama and Yamashita, 2008). Two MIS have been identified in teleosts, 17α,20β-DP (17a,20B-dihydroxy-4-pregnen-3-one and 17βS, MIH), 17a,20B,21-trihydroxy-4-pregnen-3-one, 20βS). Maturation-inducing steroids are a potent trigger oocyte maturation in many fish species, and 20βS appears to be a potential inducer of oocyte maturation in salmonids (Butts et al., 2012). The synthesis of MIH is an example of the two-cell theory, which describes the participation of two cells in sex steroid synthesis. In the oocyte growth phase, the follicular cells produce E2, which plays a role in oocyte growth and vitellogenesis. In the maturation phase, the follicular cells synthesise MIH, the key factor in successful oocyte maturation (Nagahama and Yamashita, 2008).

In the maturation phase, the oocyte completes the first meiosis, regulated by LH and MIH. Finally, MIH stimulates the synthesis of maturation promoting factor, which mediates final oocyte maturation.

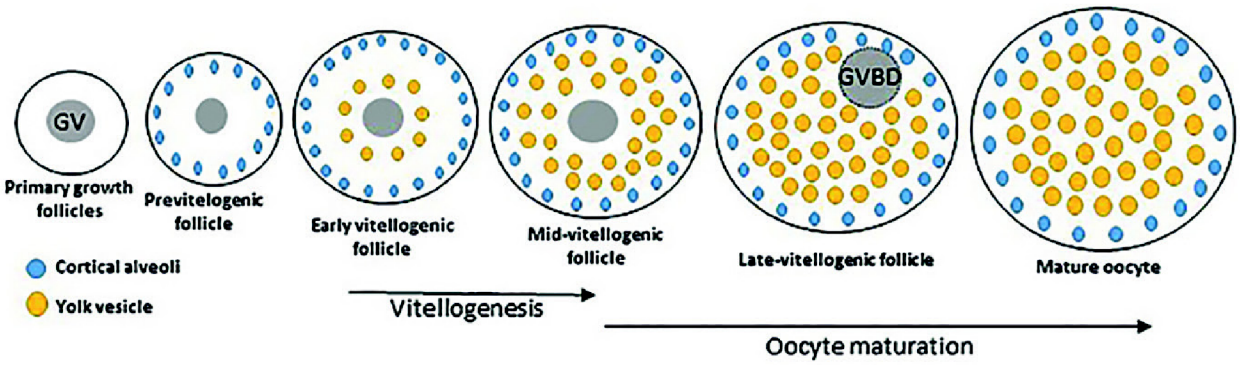

Figure 2. *A schema of oocyte developmental phases: GV: Germinal vesicle, GVBD: Germinal vesicle breakdown. Adapted from (Lubzens et al., 2010).*

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CHAPTER 1.2. SUSTAINED DRUG DELIVERY SYSTEM IN FISH AND THE POTENTIAL FOR USE OF PLGA MICROPARTICLES

Matejkova, J., Podhorec, P., 2019. Sustained drug delivery system in fish and the potential for use of PLGA microparticles: a review. Veterinarni Medicina 64, 287–293.

My share on this work was about 80%.

Veterinarni Medicina, 64, 2019 (07): 287–293 *Review Article*

https://doi.org/10.17221/161/2018-VETMED

Sustained drug delivery system in fish and the potential for use of PLGA microparticles: a review

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Citation: Matejkova J, Podhorec P (2019): Sustained drug delivery system in fish and the potential for use of PLGA microparticles: a review. Veterinarni Medicina 64, 287–293.

Abstract: Many fish species display some form of reproductive disorder in captivity. Captive fish reared in conditions outside the natural spawning environment show a failure of the pituitary to release the maturational gonadotropin luteinizing hormone thus necessitating administration of the hormone to induce spawning. A controlled sustained-release delivery system can conquer the issue of short half-life of gonadotropin releasing hormone (GnRH) in blood and avoid the necessity of using re-injections. Sustained release of GnRHa can induce long-term enhancement in semen production and multiple spawning in species with asynchronous or multiple batch group synchronous ovarian physiology. The most recent development is the incorporation of GnRHa into microparticles of biodegradable polymers that release the drug during a certain period of time ranging from days to weeks. The most attractive polymeric candidate used as a carrier for administering a pharmaceutical products is poly(lactic-co-glycolic acid); (PLGA). PLGA has excellent biodegradability and biocompatibility and is generally recognised as safe by international regulatory agencies including the European Medicines Agency and the United States Food and Drug Administration. This review describes methods of hormonal treatment in fish, highlights the advantage of sustained drug delivery system and discusses the potential of PLGA microparticles as a tool for achieving successful reproduction.

Keywords: GnRHa; reproductive dysfunction; induced spawning; aquaculture

Contents

1. Introduction

The potential to manipulate ovulation and spermiation of reared fish species, without which aquaculture would depend on broodstock, larvae, and fry from nature stock, is of great interest (Bromage 1995). Many economically relevant fish species do not breed naturally in fish farms, possibly as

Supported by the Ministry of Education, Youth and Sports of the Czech Republic – CENAKVA (Project No. CZ.1.05/2.1.00/01.0024), CENAKVA II (Project No. LO1205 under the NPU I program), NAZV (Project No. QK1810221) and GAJU (Project No. 060/2016/Z).

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https://doi.org/10.17221/161/2018-VETMED

a result of stress or environmental conditions differing from their natural habitat, leading to inadequate supplies of quality juveniles for culture (Donaldson and Hunter 1983). A constant supply of high-quality sexual products and fry is essential for profitable intensive aquaculture.

It is necessary to develop techniques for controlled reproduction in fish kept in fish farms to address complications associated with spawning synchronization, egg collection, seasonal reproduction, and reproductive dysfunction. Sustained drug release systems have enormous potential to overwhelm obstacles to successful reproduction of many fish species (Rather et al. 2013).

2. Hormonal treatment

In aquaculture, reproduction can be stimulated using manipulation of the environment or hormones (Bromage et al. 2001). The first hormones used to induce spawning in captive fish were pituitary homogenates (hypophysation) (Fontenele 1955). The efficiency of the treatment is on account of high luteinizing hormone (LH) amount. However, the major disadvantage is that pituitary homogenate used in hatcheries is generally not standardised with respect to the precise LH dose, due to the changeable LH amount in fish pituitary (Yaron 1995).

Gonadotrophin releasing hormone (GnRH) is used for stimulation of gametogenesis (Lam 1982). It has the advantage of acting at the upper level of the reproductive axis, specifically on the pituitary, supporting a more complete physiological provocation of the entire reproduction process (Duncan et al. 2003), whereas pituitary homogenates act directly on the gonads. These treatments are believed to induce an enhancement of LH production. Nevertheless, adequate spawning often does not result, due to the short life of GnRH in the blood stream. This is caused by very quick degradation of GnRH by specific endopeptidases and non-specific exopeptidases (Goren et al. 1990).

Successful approaches in the control of reproduction in a variety of many species have been reached by the administration of synthetic gonadotrophin releasing hormone analogue (GnRHa) (Crim and Bettles 1997). Chemical synthesis of GnRHa eliminates the danger of transferof infectious diseases and allows for administration of precise dosages. The process is simplified by the high grade of interspecies similarity in the GnRH peptide (Chen and Fernald 2008). The biggest advantages of GnRHa over LH preparations are higher affinity to GnRH receptors and higher resistance to enzymatic cleavage (Well et al. 1992), allowing GnRHa to remain in the blood stream for a longer time than do native forms of GnRH. As a result, longer and stronger stimulation of LH occurs (De Leeuw et al. 1988).

Administered GnRHa induces the production and release of the endogenous LH (Breton et al. 1990), which in turn stimulates final oocyte maturation and ovulation (Nagahama and Yamashita 2008) via steroidogenesis and synthesis of the maturation inducing steroid (Goetz and Garczynski 1997). GnRHa may be administered as saline injection or a sustained-release delivery system (Weil and Crim 1983). Dependent upon the GnRHa type (e.g., leuprorelin, triptorelin, buserelin), fish species, and water temperature, one GnRHa injection evokes a LH wave that remains efficient for 12–72 hours (Carrillo et al. 2000). In some fish species, a single injection of GnRHa is sufficient to promote spawning for 2–3 days post-treatment (Zakes and Demska–Zakes 2005), but re-injections are frequently essential to provoke long lasting LH release and promote complete maturation and reproduction (Pankhurst et al. 1986; Dabrowski et al. 1994; Slater et al. 1995). Multiple injections are stressful and often increase mortality of valuable broodstock. Stress may, in itself, inhibit the reproductive process and induce pathologies and even death (Schreck et al. 2001).

3. Delivery systems used for regulation of fish reproduction

Long acting preparations constitute a frontier area of science. These technologies have considerable benefits compared to conventional administration, containing controlled release, low toxicity, and better efficacy and convenience (Lin et al. 2014). It has been demonstrated that GnRHa has a half-life of less than 30 min in the blood stream (Gothilf and Zohar 1991). In contrast, the sustained release of GnRHa increases plasma GnRHa concentrations that may last for several weeks (Mylonas et al. 1995). Sustained administration of GnRHa efficiently induces a sustained LH release. The induced endogenous LH release stimulates the go-

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nads more effectively than externally administered LH (Mylonas and Zohar 2000). Sustained delivery of GnRHa is advantageous compared to conventional dosage forms (Mylonas et al. 2007). Particularly in fishes with asynchronous ovarian development, sustained administration of GnRHa stimulates a long lasting elevation of plasma LH that causes multiple spawnings (Crim et al. 1988; Carrillo et al. 2000).

The first long acting preparation for the manipulation of spawning in fish used was cholesterol (Weil and Crim 1983; Carolsfeld et al. 1988). The agent was imbedded in cholesterol pellets with included cellulose to slow the release. Cholesterol pellets with imbedded mammalian GnRHa (mGnRHa) released an initial burst. After this initial burst, a sustained release follows that may last for over 28 days (Sherwood et al. 1988). The disadvantage of this system is a significant variation in GnRHa release from individual pellets (Carolsfeld et al. 1988). Moreover, there were concerns that cholesterol as an active biomolecule and a precursor to steroid hormone synthesis may influence gonad function (Mylonas and Zohar 2000). On the other hand, administration of cholesterol pellets is simple, and they can be stored without loss of bioactivity at room temperature for at least 4 weeks (Garcia 1996). Its positive effect on induction of maturation was confirmed in many species of fish including rainbow trout *Oncorhynchus mykiss* (Crim et al. 1983), herring *Clupea harengus pallasi* (Carolsfeld et al. 1988), sea bass *Dicentrarchus labrax* (Almendras et al. 1988), milkfish *Chanos chanos* (Marte et al. 1988), Pacific white snook *Centropomus viridis* (Ibarra-Castro et al. 2017), and beluga *Huso huso* (Aramli et al. 2017).

Following the success of cholesterol pellets containing mGnRHa, commercially available cholesterol pellets containing salmon GnRHa (sGnRHa) were developed (Ovaplant, Syndel International). With this preparation, 40–60% of the sGnRHa is released during the first day, and the rest of the drug is released over the ensuing 7–21 days (U.S. Fish & Wildlife Service 2016). The preparation was successfully used in empurau *Tor tambroides* (De Silva et al. 2004), hybrid catfish *Clarias microcephalus* × *C. gariepinus* (Abol-Munafi et al. 2006), Atlantic salmon *Salmo salar* (Anderson et al. 2017) and inpacu *Piaractus mesopotamicus* (Kuradomi et al. 2017).

A non-degradable co-polymer of ethylene and vinyl acetate (EVAc) is also used as a sustained delivery system (Rhine et al. 1980). The EVAc is produced in the shape of solid implants that are administered intramuscularly (Brown et al. 1986). The release rate of EVAc implants can extend from 15 days (Gothilf 1990) to 5 weeks, depending on the structure (Zohar 1996). EVAcs are simple to manufacture, and batches of 200–500 implants can be produced in a single preparation, minimizing GnRHa content variation among implants (Mylonas and Zohar 2000). The implants remains useable for up to three years when stored dried at –20 °C (Zohar and Mylonas 2001). The EVAc systems ability to induce multiple ovulation was confirmed in European sea bass at 60 µg/kg (Fornies et al. 2001), turbot *Scophthalmus maximus* at 25 µg/kg (Mugnier et al. 2000), in Atlantic bluefin tuna *Thunnus thunnus* at 40–80 µg/kg (Rosenfeld et al. 2012), and in great amberjack *Seriola dumerili* at 50 µg/kg (Jerez et al. 2018). These results may be attributed to long term elevation of plasma LH caused by long acting release of GnRHa (Fornies et al. 2001).

4. Poly(lactic-co-glycolic acid) microparticles

Microparticle is a dosage form that can be used for delivering of drugs by many ways and efficaciously manage the release of agent (Donbrow 1991; Birnbaum and Brannon-Peppas 2004). The name microparticle refers to a particle of diameter 1–1000 µm (Birnbaum and Brannon-Peppas 2004). Microparticles can be extremely helpful to protect the encapsulated agent against enzymatic degradation. Other advantages of microparticles are the possibility of controlled release of the agent, local delivery of the incorporated agent over intervals ranging from a few hours to several weeks, and simple administration compared to normally used forms of long-acting preparations, such as implants that are often used in aquaculture (Siepmann and Siepmann 2006).

A large range of natural as well as synthetic biodegradable polymers have been examined for use as matrix for microparticles (Hoffman 2008). Among them, the thermoplastic aliphatic poly(esters) as for example poly(lactic-co-glycolic acid); (PLGA) have caused concern owing to their extremely good tissue biocompatibility, biodegradability in the body (Lewis 1990) and show high flexibility in the en-

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capsulation of different agents. Bacterial (Altun et al. 2010), viral (Adomako et al. 2012) and parasitic antigens (Harikrishnan et al. 2012) encapsulated in PLGA were successfully used in fish and offered effective protection against pathogens.

Majority of biodegradable polymers decompose via hydrolysis to biologically tolerable and progressively minor compounds. In PLGA, the polymer eventually breaks down into lactic and glycolic acid, which enter the Krebs cycle. Afterwards it is further decomposed to carbon dioxide and water (Houchin and Topp 2009).

Microspheres of PLGA and other polymers are usually formed by solvent evaporation (Wakiyama et al. 1981; Bai et al. 2001; Ruan and Feng 2003). Frequently a double emulsion is used in which medicament that will be encapsulated is primarily dissolved in water. The aqueous phase is dissipated in an organic solvent (Dichloromethane) that includes the degradable polymer, and a water/oil emulsion is formed. Dispersion of the first emulsion in a steady aqueous medium, normally with poly(vinyl alcohol) as stabilizer, forming the final water/oil/water double emulsion. Microspheres are produced when the dichloromethane vaporizes and the polymer solidifies, incorporating the encapsulated pharmaceutical agent (O'Donnell and McGinity 1997).

5. Factors affecting drug release

Drug release kinetic is affected by the form of the matrix in which the active substance is incorporated and the chemical characteristics of the polymer and the medicament (Freiberg and Zhu 2004). A consistent release rate over time is desirable but release profiles may consist of an initial burst of medication released from the microparticle uppermost layer followed by a constant sustained release that depends on diffusion as well as on decomposition (Mogi et al. 2000).

The method of particle fabrication governs the incorporation and release of the therapeutic agent. A complex range of elements containing the kind of polymer, its molecular weight, the co-polymer structure, the nature of pharmaceutical ingredients incorporated to the microparticle mixture (therapeutic agent stabilization), and microparticle size can affect the release rate (Raman et al. 2005).

The kind of polymer employed in microparticle production and the process in which it breaks down surely affects therapeutic agent release kinetics (von Burkersroda et al. 2002). Bulk-eroding polymer microspheres such as PLGA are frequently distinguished in release of up to 50% of the complete therapeutic agent load over the first few hours after application. This initial burst is succeeded by slow release and occasionally a third stage during which the remainder is released rapidly due to the polymer decomposition (O'Donnell and McGinity 1997).

Both acidic and strongly alkaline media accelerate degradation of PLGA (Holy et al. 1999). Nevertheless, the contrast between neutral and slightly acidic environment is less significant because of autocatalysis by the carboxylic end groups (Zolnik and Burgess 2007). Enzymatic degradation also influences drug release. Poly(lactic-co-glycolic acid) is decomposed firstly via hydrolysis, but it has also been proposed that enzymatic decomposition can affect the drug release from the microparticles. Lack of uniformity *in vivo* makes it complicated to compare the contribution of obtainable enzymes to the degradation process (Cai et al. 2003).

6. Conclusions

Sustained delivery systems for GnRHa have shown to be effective methods for hormonal regulation of fish spawning. Apart from the type of hormonal treatment, the form of its administration is a major factor influencing the efficacy of the preparation. In the case of conventional drug release treatment, the hormonal preparation is dissolved in physiological saline, but a significant obstacle is the rapid enzymatic degradation of the administered peptide reducing its efficacy (Goren et al. 1990). Therefore, multiple administration is required to maintain the effective level of the preparation, otherwise the plasma concentration of the peptide falls below the therapeutic level (Schreck et al. 2001). The development of advanced drug delivery system opens new possibilities for effective administration of biologically active substances in the structure of microparticles enabling the release of bounded hormonal substances at the desired dose and for a set time.

Drug delivery using PLGA is an interesting field with extensive potential for biomedical investigation. They are easily produced and can preserve the agent from degradation and increase its stability. Microparticles of PLGA can increase the effica-

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cy of treatment through long acting of the drug. It is approved by the US Food and Drug Administration and European Medicine Agency in diverse long acting systems. PLGA microparticles show potential as a long acting preparation in fish reproduction.

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Received: November 29, 2018
Accepted after corrections: May 28, 2019

1.3. Objectives of the dissertation

The aim of this dissertation was to test and verify an innovative method of hormonal stimulation using PLGA microparticles as a system for sustained release of a synthetic analogue of mammalian gonadotropin-releasing hormone in several economically important fish species and elaborate a study reviewing methods of hormonal treatment in fish, and discuss the potential of PLGA microparticles in fish reproduction (Chapter 1). Chapter 2 present information about the formation of PLGA microparticles and provides overview about their physical, chemical, analytical, and pharmaceutical properties. Chapters 3 and 4 study the impact of PLGA microparticle treatment on the reproductive output of females. Chapter 3 provides information about the effect of two types of PLGA microparticles on ovulation in peled while Chapter 4 is focused on the impact of the treatment on ovulation in pikeperch. Chapters 5, 6, and 7 address the impact of the extended PLGA treatment on spermiation in northern pike (Chapter 5), sterlet (Chapter 6) and Bala shark (Chapter 7).

CHAPTER 2

PRE-FORMULATION DESIGN OF SUSTAINED-RELEASE GNRHA-LOADED PLGA MICROSPHERES AND ASSOCIATED FORMULATIONS FOR CONTROLLING REPRODUCTION IN AQUACULTURE

Holická, M., Vysloužil, J., Kubová, K., Muselík, J., Radinova, E., Vetchý, D., Kotolová, H., Hammer, T., Mašek, J., Podhorec, P., Knowles, J., 2021. Pre-formulation design of sustained-release GnRHa-loaded PLGA microspheres and associated formulations for controlling reproduction in aquaculture. Acta Poloniae Pharmaceutica 78, 801–812.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis
https://www.ptfarm.pl/wydawnictwa/czasopisma/acta-poloniae-pharmaceutica/2389/2392

My share on this work was about 5%.

Acta Poloniae Pharmaceutica – Drug Research, Vol. 78 No. 6 pp. 801–812, 2021
DOI: 10.32383/appdr/146287
Received 16 December 2021, Received in revised form 21 January 2022, Accepted 31 January 2022

ISSN 2353-5288
Polish Pharmaceutical Society

PHARMACEUTICAL TECHNOLOGY

PRE-FORMULATION DESIGN OF SUSTAINED-RELEASE GnRHa-LOADED PLGA MICROSPHERES AND ASSOCIATED FORMULATIONS FOR CONTROLLING REPRODUCTION IN AQUACULTURE

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Abstract: Poly(lactide-co-glycolide) PLGA microparticles represent an efficient and modern tool to encapsulate peptide drugs, which enables their administration to live organisms with the benefit of prolonged-release. One area that could make the best of this opportunity is fish breeding in aquaculture. The presented study was centered on the formulation of gonadotropin-releasing hormone (GnRH) analog loaded PLGA microparticles intended for fish breeding augmentation, using the double emulsion evaporation method. In this initial experiment, the influence of several input variables (drug, PLGA type, emulsifier concentration, gelatine concentration in internal phase) on observed parameters (morphology, particle size, drug content, drug release, resuspension index) was evaluated. It was found that at lower emulsifier concentration, the particle size is apparently lower (8.54 ± 6.13-10.36 ± 4.65 vs. 25.56 ± 18.86), which is more advantageous in injection administration. Encapsulation efficiency ranged from 39.13 ± 8.85 to 75.30 ± 8.83, favoring lower emulsifier concentration, while resuspension index (70.99 ± 6.47%) suggested the possibility of longer-term administration. Dissolution tests revealed prolonged release for ten days, with most of the drug released in 72-96 hrs. A follow-up study discerning polymer type/gelatine concentration was suggested. Accompanying studies of imaging agent coumarin-6 encapsulation for eventual distribution imaging and metoclopramide base drug release for potential adjuvant use were also successfully realized.

Keywords: GnRH analogs, microparticles, solvent evaporation, gelatine, sustained drug release, fish reproduction

One of the existing approaches to increase the yield of the reproductive process in fish farming is hormone therapy, used to induce both ovulation and spermiation (1). For this purpose, gonadotropin-releasing hormone (GnRH) and its analogs (GnRHa), mainly in the form of agonists, can be administered. Numerous studies reporting their positive effect on various species have been published (2, 3). Moreover, dopaminergic agents prevent neuropeptides' synthesis or block their release from the pituitary gland in fish species with dopamine inhibition (4, 5). Therefore, GnRH or its analogs can be combined with dopaminergic inhibitors like metoclopramide or domperidone to improve the therapy effect further (1).

However, this method of use also brings challenges based on pharmacological/pharmaco-technological aspects. If administered orally, GnRH analogs are eliminated by harsh conditions in the gastrointestinal tract (GIT). If administered parenterally as simple suspension, unstable water-soluble GnRH analogs remain unprotected against enzymes, and their half-life is relatively short (6). It can result in the need for repeated administration. However, it

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is well-known that recurring handling of the treated population causes stress, and it can negatively affect the quantity and quality of gametes (7). Therefore, it is logical to explore possibilities for encapsulating such agents. The solution can be seen in polymeric microparticle carriers. With an appropriate polymer as a carrier, microparticle dosage forms can offer protection against the environment, decrease the single-dose amount, systemic side effects, reduce administration frequency, and the possibility of dose dumping, resulting in so-called controlled drug delivery. It effectively means that after an administration of such a dosage form, the drug is delivered in therapeutic dose for a prolonged time interval without the need for re-administration (8). This concept could also be advantageous in fish, especially in breeding populations (9).

Several polymers were examined as microparticle carriers in fish, including chitosan (10) or copolymer of ethylene and vinyl acetate (11). Very promising are polyesters, such as poly(lactide-*co*-glycolide) (PLGA) and poly(L-lactide) (PLLA), previously successfully used as antigen carriers in fish (12, 13). They can release an active agent over a more extended period, and due to their biodegradability and biocompatibility, they are very well tolerated systems (14), FDA approved for human use. Therefore, it is clear they make a strong candidate for use in fish reproduction as a GnRHa carrier (15), especially in cases when a combination of PLGA with some synthetic GnRH analogs has not been tested yet.

Before a final formulation design is devised, the pre-formulation study is usually performed to estimate the preparation system behavior and set the basic parameters. Therefore, the main objective of the presented paper is the formulation preparation of GnRHa-loaded PLGA microparticles and their subsequent evaluation. To our knowledge, the selected combination of PLGA and alarelin as GnRHa was not previously studied in fish. Emphasis was placed on prolonged drug release achievement and verification of the microparticles' applicability to a living organism concerning the pharmaceutical-technological parameters to build a foundation for further *in vivo* testing. Because the chosen preparation method is essentially simple and with certain analytical backgrounds quite feasible outside the pharmaceutical technology laboratory, an explanation is given at individual evaluations to clarify their practical impact on dosage form administration. Last but not least, a simple metoclopramide base dissolution study was included to complete the outline of possible adjuvant treatment, and the design of fluorescent microparticles was suggested for eventual distribution and immunology studies.

MATERIAL AND METHODS

Materials

The main drugs used for the study were GnRH analogs alarelin acetate and leuprorelin acetate (both APExBIO, USA). Coumarin-6 (Sigma Aldrich, USA) served as a model substance for fluorescence study. Gelatine (Sigma Aldrich, USA) was used as an inner aqueous phase thickener to increase a microparticle core density. As a polymer carrier poly(lactide-*co*-glycolide) acid (PLGA) polymers were used: Resomer® RG 504S (50:50 lactide:glycolide ratio), Resomer® RG 653H (65:35 ratio) and Resomer® RG 753S (75:25 ratio; all Evonik, Germany). Dichloromethane (Penta, Czech Republic) was the organic solvent used for the oil phase, polyvinyl alcohol (PVA; Mw 31.000–50.000; 98–99% hydrolyzed) (Sigma Aldrich, USA) acted as an emulsifier, and sodium chloride (Dr. Kulich Pharma, Czech Republic) as an inorganic salt and osmolyte. Ball-milled (PM 100, Retsch, Germany) metoclopramide (Carbosynth, United Kingdom) intended as an adjuvant was used in the form of standard suspension, whose drug release was also evaluated. Phosphate buffer solution pH 7.0 for *in vitro* release consisted of 5 g of potassium dihydrogen phosphate and 11 g of potassium hydrogen phosphate (both Dr. Kulich Pharma, Czech Republic) in 900 mL of purified water. All materials were of Ph. Eur. quality.

Microparticles preparation

Alarelin and leuprorelin-loaded microparticles were obtained by the double emulsion ($water_1$/oil/$water_2$; w_1/o/w_2) solvent evaporation method. Precisely 800 mg of polymer were dissolved in 5 mL of dichloromethane, representing the oil phase (o). For the inner aqueous phase (w_1) formation, 10 mg of alarelin or leuprorelin were weighed and dissolved in 1.5 mL of gelatine solution (2.5 or 9.09%). The drug-gelatine solution was added to the oil phase. This mixture was pre-mixed by vortex (Lab Dancer, IKA-Werke GmbH & Co. KG, Germany) for 30 seconds. Then the mixture was stirred by homogenizer (T25 basic, IKA-Werke, Germany) at 11 000 rpm for 60 seconds to form a simple w/o emulsion. The drug-gelatine solution was dispersed in the polymer solution. Gelatine solution acted not only as the drug solvent but also as a thickener, increasing the inner water phase viscosity (16). If simple water is used instead of gelatine, the stability of the primary emulsion

would be decreased, and the encapsulation efficiency would be an order of magnitude lower (17).

Consequently, 12 mL of 1% PVA solution were added to the w/o emulsion (besides the A653-G9$_{0.1}$ sample, where 0.1% PVA was used for pre-mixing), and the mixture was stirred by homogenizer at 11 000 rpm for 60 seconds. PVA serves as an emulsifier, which improves the dispersion of the emulsion phases. Finally, after the pre-mixing step, the concentrated double emulsion was poured into a 200 mL solution consisting of 0.1% PVA/2% NaCl solution, basically resulting in diluted $w_1/o/w_2$ emulsion. NaCl is osmotically active, slowing the leakage of the hydrophilic drug from the internal w_1 phase. The organic solvent was then evaporated under the mechanical stirrer (Heidolph RZR 2021, Sigma Aldrich, USA) at 450 rpm for 120 minutes, and thus the droplets slowly solidified into spherical particles. The resulting micro-suspension was passed through a 250 µm mesh sieve to remove agglomerates, and microparticles were collected by centrifugation (EBA 20, Hettich, Germany). The obtained particles were resuspended, frozen, and lyophilized (L4-55 PRO, Gregor Instruments, Czech Republic). The lyophilization was carried out until probe and sample temperature evened out and then for another eight hours to ensure secondary drying.

The list of prepared samples with their formulation parameters is summarized in Table 1. The first letter in the sample nomenclature denotes drug (A - alarelin, L - leuprorelin, C - coumarin-6), followed by a number specifying PLGA type (504, 653, 753), ended with G for gelatine (if used) and its concentration in% (2.5, 9.09). Eventually, the last number in the lower index denotes PVA concentration for pre-mixing. If not present, 1% PVA was used. Sample containing coumarin-6 was prepared similarly but using simple w/o solvent evaporation method. The samples A504-G2.5 and A653-G9$_1$ consisted of three batches, four runs each, whereas the samples A653-G9$_{0.1}$ used 0.1% PVA for the pre-emulsion step; L653-G9, 753-G9, and C753 consisted of one batch only from 4 runs. A blank sample (753-G9) was prepared under non-pyrogenic conditions (depyrogenation in acetone (Penta, Czech Republic)), followed by heating at 250°C for 90 minutes, using water for injection and laminar box for preparation).

Microparticles evaluation

Drug content analysis

The alarelin and leuprorelin content in PLGA microparticles was determined by high-performance liquid chromatography (HPLC). Firstly, the PLGA microparticles were dissolved in acetone, and the resulting solution was mixed with phosphate buffer of pH 7.0 in a 1 : 1 ratio (v/v). This mixture was filtered through a 0.45 µm membrane filter. The content was then quantified by the HPLC (Agilent 1100, Agilent Technologies, USA) involving NUCLEODUR 100-5 CN-RP (150 mm × 4.6 mm, 5 µm) column. A binary mixture of acetonitrile: 20 mM H_3PO_4 (16 : 84, v/v) was used as a mobile phase at 0.8 mL/min flow rate with a temperature set at 30°C, injected sample volume of 20 µL and detection wavelength of 220 nm.

The coumarin-6 content from sample C753 was determined by a UV/Vis spectrometer (Lambda 25, Perkin Elmer, USA) at 450 nm. The samples were prepared by dissolving 100 mg of microparticles in 10 mL of dichloromethane and filtered through a 0.22 µm membrane filter. This procedure was conducted in triplicate.

Mean values and standard deviations (SD) were expressed from the obtained values. Encapsulation efficiency (%; EE) [1], drug load (%; DL) [2], and practical yield (%) [3] were also determined by using the equations below (18, 19, 20).

$$EE = \frac{w_1}{c_t} \times 100\,[\%] \qquad [1]$$

Table 1. The microparticles samples and their formulation/process characteristics

Sample*	PLGA polymer**	Drug, amount	Gelatine solution - volume (mL), concentration (%)	PVA (%)	Method
753-G9	753S	-	1.5, 9.09	1.0	$w_1/o/w_2$
A504-G2.5	504S	Alarelin, 10 mg	1.5, 2.5	1.0	$w_1/o/w_2$
A653-G9$_1$	653H	Alarelin, 10 mg	1.5, 9.09	1.0	$w_1/o/w_2$
A653-G9$_{0.1}$	653H	Alarelin, 10 mg	1.5, 9.09	0.1	$w_1/o/w_2$
L653-G9	653H	Leuprorelin, 10 mg	1.5, 9.09	1.0	$w_1/o/w_2$
C753	753S	Coumarin-6, 500 µg	-	1.0	o/w

*A - alarelin; L - leuprorelin, G - gelatine; **the first two numbers express the ratio of lactic acid monomers in PLGA, the third number represents the PLGA viscosity (dL/g), S - ester terminated, H - free carboxylic acid

$$DL = \frac{w_1}{w_2} \times 100\,[\%] \quad [2]$$

$$Yield = \frac{w_2}{w_t} \times 100\ [\%] \quad [3]$$

where: w_1 represents the actual weight of the drug in microparticles; c_t is the theoretical amount of drug; w_2 is the total weight of prepared microparticles; w_t is the theoretical yield (total amount of drug and polymer used for the microparticle preparation).

Scanning electron microscopy (SEM)

The morphology and properties of samples were evaluated using scanning electron microscopy (MIRA3, Tescan Orsay Holding, Czech Republic) equipped with a secondary electron detector. Using carbon conductive double-sided adhesive tape, a sample was mounted on an SEM specimen stub (Agar Scientific, United Kingdom). Microparticles were coated by a layer of gold (20 nm) to eliminate charging artifacts using the metal sputtering coating method with argon atmosphere (Q150R ES Rotary-Pumped Sputter Coater/Carbon Coater, Quorum Technologies, United Kingdom). SEM images were obtained at an accelerating voltage of 3 kV.

Optical microscope analysis

Morphological properties of the prepared microparticles (sphericity factor, equivalent diameter) were evaluated using an optical microscope (Nikon Eclipse E200, Nikon, Japan) coupled with a camera (72AUC02 USB, The Imaging Source, Germany). Randomly selected 200 microparticles were evaluated by computer software (NIS-Elements AR 4.0, Nikon, Japan). An image of every sample was also taken.

Sphericity factor (SF) is a dimensionless unit describing particles shape. Equivalent diameter (ED) determines the circle diameter having the same area as the object observed. Both parameters can be determined according to the equation below:

$$SF = \frac{4\pi A}{p^2} \quad [4]$$

$$ED = \sqrt{\frac{4\,A}{\pi}}\,[mm] \quad [5]$$

where: A represents area in mm^2 and p is microparticles perimeter in mm (21).

Laser diffraction

Laser diffraction was performed using the laser particle size analyzer to determine size distribution (LA-960, Horiba Scientific, Japan). The real refractive index was set to 1.6. The imaginary refractive index was neglected. Sufficient sample weight was resuspended in 1 mL of purified water to induce signal. Resuspended samples were placed into the device, measured immediately, and analyzed for volume-based size distribution. The measurements were performed in triplicate. The results were expressed as median, mean values with SD, and the diameters at the 10th, 50th, and 90th percentiles of the cumulative undersize plot (D10, D50, D90).

Confocal microscope analysis

The water suspension of the C753 sample (10 mg/mL) was placed into µ-Slide 8 Well (Ibidi GmbH, Gräfelfing, Germany). After sedimentation, microparticles were observed using a confocal microscope (Leica SP8, Leica, Germany). A 458 nm wavelength laser was used for the excitation, and a hybrid emission detector (HyD) was set to 530-560 nm. The confocal microscopy images were acquired with a Leica objective HCX PL APO 63x.

In vitro *release studies*

In vitro alarelin/leuprorelin release in buffer solution: *In vitro* release testing of alarelin (time course ten days) was performed in Franz cells connected in series at 5 ± 0.5°C without stirring. Exactly 20 mg of microparticles were placed into a cell containing 20 mL of phosphate pH 7.0 buffer solution. The release medium was sampled (1.0 mL) regularly every 24 hours, followed by a complete replacement of the dissolution medium in cells. All measurements were performed with six units. Alarelin concentration was quantified by HPLC using the method stated in the *Drug content analysis* section. The dissolution test of leuprorelin was conducted in the same fashion except that 50 mg of sample were weighed into 30 mL of pH 7.0 buffer (22). The pH value varies in different tissues and fish species (23). However, the flesh pH is very often close to pH 7.0 (24). Therefore it was chosen as a reasonable compromise.

In vitro alarelin release in agar: The method was based on the adjusted procedure used in the release study of GnRHa-loaded polyanhydride microparticles (25). The selected samples A504-G2.5 and A653-$G9_1$ were tested in agar gel under three conditions. Exactly 50 mg of microparticles were suspended in 1%/0.4 mL, 1%/0.8 mL, or 2%/0.4 mL agarose solution in a glass vial, cooled down to let the agarose solidify, and then another

400 µL of the respective agarose solution were added to form a covering layer. When the cover layer had solidified, 5 mL of phosphate buffer was added. Precisely, 2 mL were taken after 23, 49, 73, 96, 168, and 240 hours and they were filtered through a 0.22 µm membrane filter. The buffer residue was wholly removed, the vials were washed with 0.5 mL of buffer, and 5 mL of fresh buffer was added. *In vitro* experiments were run at 5°C, and for each sample, they were performed in triplicate. The fish body temperature varies significantly among the species, mainly depending on their habitat (26). The chosen value represents some seawater and freshwater species living in cold waters (27).

Metoclopramide suspension: A biphasic dissolution setup was used to evaluate metoclopramide drug release. The metoclopramide suspension (0.5 mL) was pipetted into a cell containing 10.0 mL of octanol and 9.5 mL of pH 7.0 phosphate buffer. Samples from the octanol phase (0.2 mL) were sampled for ten days, and the volume of the withdrawn dissolution medium was removed by pure octanol. Dissolution was performed in triplicate. Metoclopramide assessment in dissolution medium was quantified by HPLC (Agilent 1100, Agilent Technologies, USA) on xBridge C18 column with a mobile phase consisting of acetonitrile: 20 mM H_3PO_4 (15 : 85, v/v) at the flow rate of 1 mL/min and spectrophotometric detection under the wavelength of 272 nm.

Resuspension index

Resuspension index (RI) expresses the percentage of resuspended microparticles remaining resuspended in a medium after a given time. Exactly 30 mg of microparticles were placed in a 1.5 mL Eppendorf tube, 1 mL of distilled water was added, and it was vortexed for 1 minute. This suspension was left to stand for 10 minutes to allow the aggregated particles to sediment. The formed suspension was gently collected into a centrifuge tube. This procedure was performed in a total of three consequent repetitions. The collected suspension was centrifuged at 1500 rpm for 10 minutes, and the supernatants were removed. Resuspended and aggregated fractions were lyophilized and weighted. Triplicate determinations were performed for the 753-G9 sample, and the result RI was calculated as:

$$RI = \frac{m_r}{m_r + m_a} \times 100 \ [\%] \qquad [6]$$

where: m_r expresses the resuspended mass and m_a the aggregated mass.

Pierce chromogenic endotoxin test

Each sample in 200 mg was dispersed in 2 mL of endotoxin-free water. The supernatant was collected from the settled suspensions, and endotoxin levels were tested using the Pierce test (Pierce Chromogenic Endotoxin Quant Kit, Thermofisher Scientific, USA).

RESULTS AND DISCUSSION

Alarelin and leuprorelin-loaded microparticles were successfully formulated by the double emulsion ($w_1/o/w_2$) method using the pre-mixing step. The creation of the concentrated double emulsion prevented droplets' coalescence during the process. It resulted in final microparticles with particle size in tens of microns, considered optimal for non-problematic injectability (28). Based on data available in scientific literature, the PLGA microparticles prepared without this step exhibited significantly bigger particle sizes in hundreds of microns (29). Coumarin-6-loaded particles were also successfully prepared by the o/w method.

Encapsulation process and yield

Table 2 shows the yield and drug content analysis results. These parameters give information about the efficiency and loss rate of the preparation process. In this case, it provided a satisfactory yield, as the lowest value was 73.97% for L653-G9. The highest was found in Resomer® 653H sample A653-$G9_1$ (83.14 ± 2.54%). In 1% PVA alarelin samples, the EE and DL analysis turned out to be in favor of Resomer® 504 with EE of 46.56 ± 6.18% and DL 0.55 ± 0.07%. The differences from particles prepared with Resomers® 653H can be attributed to the higher viscosity of the Resomer® 504 (30). Higher polymer viscosity produces lower yield through process losses (adherence to equipment). On the other hand, higher viscosity increases drug entrapment, resulting in higher values of EE and DL (31). Nevertheless, since the values overlap when SD is considered, the EE and DL of both samples could be seen as comparable, and further investigation will be required. Comparing A653-$G9_1$ and A653-$G9_{0.1}$ samples clearly shows that the lower PVA concentration used for the pre-mixing step resulted in significantly higher EE and DL. This observation is very probably related to their higher mean size. The overall surface is lower, and the drug thus has less opportunity to leak during the preparation process (32). However, a higher mean size may cause problematic administration via syringe; therefore, a final formulation may be understood as a compromise.

Table 2. Encapsulation efficiency, drug loading, yield, mean size and sphericity, and their respective standard deviations.

Sample	EE (%)	DL (%)	Yield (%)	Mean size (μm)	Sphericity factor
753-G9	-	-	75.36	6.25 ± 2.51	0.999 ± 0.001
A504-G2.5	46.56 ± 6.18	0.55 ± 0.07	77.01 ± 0.78	8.54 ± 6.13	0.973 ± 0.027
A653-G9$_1$	39.13 ± 8.85	0.41 ± 0.09	83.14 ± 2.54	10.36 ± 4.65	0.999 ± 0.001
A653-G9$_{0.1}$	75.30 ± 8.83	0.80 ± 0.09	75.32	25.56 ± 18.86	0.999 ± 0.001
L653-G9	58.98 ± 1.77	0.62 ± 0.02	73.97	8.72 ± 4.31	0.999 ± 0.002
C753	91.50 ± 0.96	0.06 ± 0.00	74.30	7.19 ± 2.80	0.998 ± 0.003

EE = encapsulation efficiency; DL = drug loading

The leuprorelin sample L653-G9 showed a favorable result with EE of 58.98 ± 1.77%, compared to the corresponding alarelin sample. Excellent EE was reached in the sample C753 with the value of 91.50 ± 0.96%, which resulted from the coumarin-6 lipophilic character (33).

Morphology and particle size

SEM analysis (Figure 1) showed excellently spherical particles for each prepared sample. It revealed a smooth surface without cracks, and it hinted at lower porosity with decreasing glycolic acid content in a polymer (34). Resomer® 504S (Figure 1 - C, D) provided particles with numerous pores; meanwhile, Resomer® 753 (Figure 1 - A, B) yielded particles without visible pores. The images also suggested partially hollow particles, mainly in the Resomer® 504S sample. By estimation, particle size ranged in the interval 1-30 μm. It means polydisperse particles with relatively heterogeneous size distribution with included agglomerates. Figure 1 - G, H also indicates bigger particles in the sample A653-G9$_{0.1}$ pre-mixed with 0.1% PVA than in the corresponding 1% PVA sample, confirming the

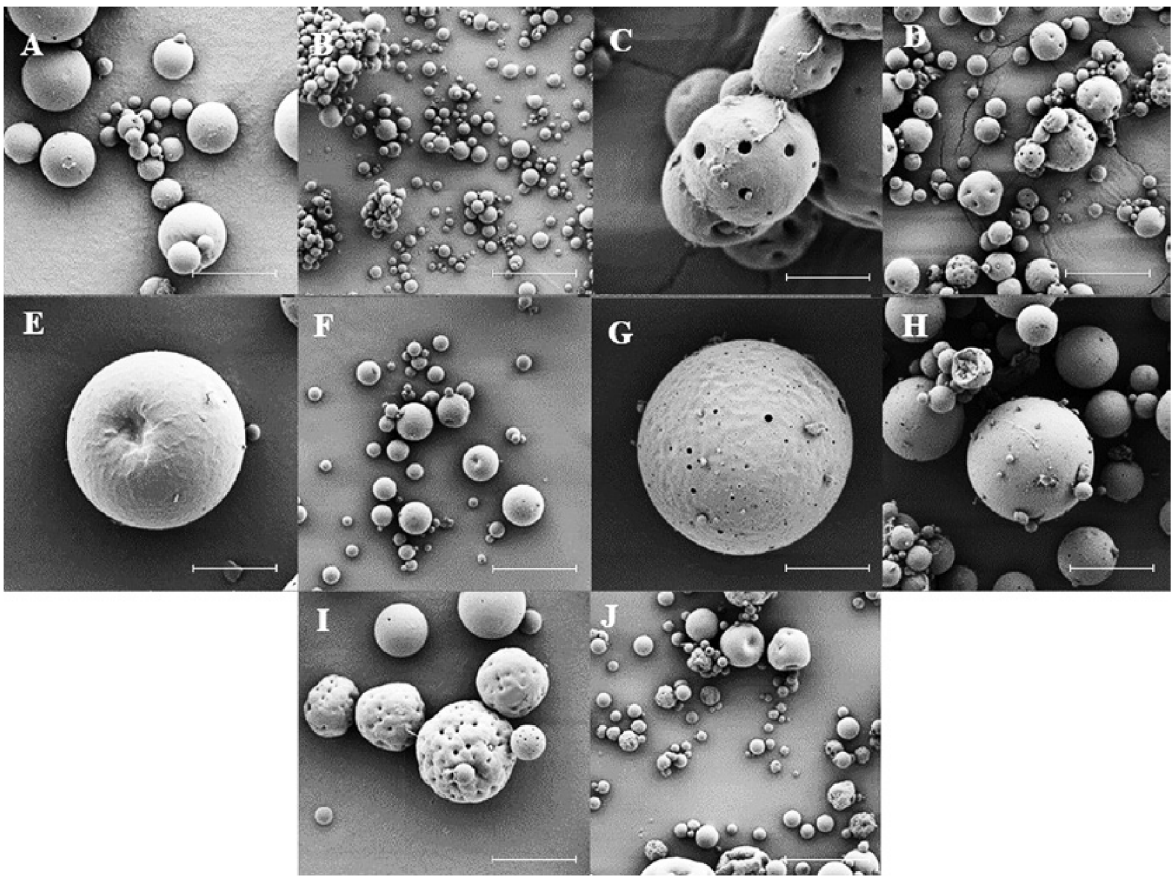

Figure 1. SEM analysis – A) 753-G9 sample (bar = 10 μm); B) 753-G9 sample (bar = 50 μm); C) A504-G2.5 sample (bar = 10 μm); D) A504-G2.5 sample (bar = 50 μm); E) A653-G9$_1$ sample (bar = 10 μm); F) A653-G9$_1$ sample (bar = 50 μm); G) A653-G9$_{0.1}$ sample (bar = 10 μm); H) A653-G9$_{0.1}$ sample (bar = 50 μm); I) L653-G9 sample (bar = 10 μm); J) L653-G9 sample (bar = 50 μm)

fundamental influence of PVA concentration on particle size (35).

Optical microscope analysis followed, allowing particle size and sphericity measurement (Table 2). Images of the prepared microparticles are shown in Figure 2. In general, every prepared sample consisted of highly spherical particles as the sphericity factor ranged between 0.973 ± 0.027 and 0.999 ± 0.001. For comparison, a pellet sphericity factor of 0.9 is usually stated as sufficient (36). This feature is highly desirable because high sphericity means better flow properties of the dry powder. It also gives a large surface for eventual resuspension in the injection medium. The particle size prepared using 1% PVA took values from 6.25 ± 2.51 to 10.36 ± 4.65 µm, suggesting a parenterally applicable dosage form into fish organisms (37, 38). The sample A653-$G9_{0.1}$ pre-mixed with 0.1% PVA exhibited a two times higher particle size (25.56 ± 18.86 µm), confirming observations and conclusions reached in previous paragraphs. In addition to the optical microscope analysis, for the C753 sample, the confocal microscope analysis was performed to prove the sample functionality. Figure 3 shows that the particle manifested detectable fluorescence. This feature can be used for tissue deposition studies *in vivo* to confirm correct administration or in immunology studies.

Finally, laser diffraction was performed to complete the particle size examination (Figure 4 and 5, Table 3). The median was approximately 20 µm for all 1% PVA samples. Diameter values on cumulative % were ranged from 7.8 to 13.2 µm (10%), 15.4 µm to 26.2 µm (50%) and 41.4 µm to 64.2 µm (90%). The laser diffraction also confirmed previous observations that the sample A653-$G9_{0.1}$ with a median of 35.7 µm provides a significantly higher particle size. Wide distribution interval (Figure 4, 5) and higher SD indicate polydisperse samples. Compared to the optical microscope analysis, the particle size

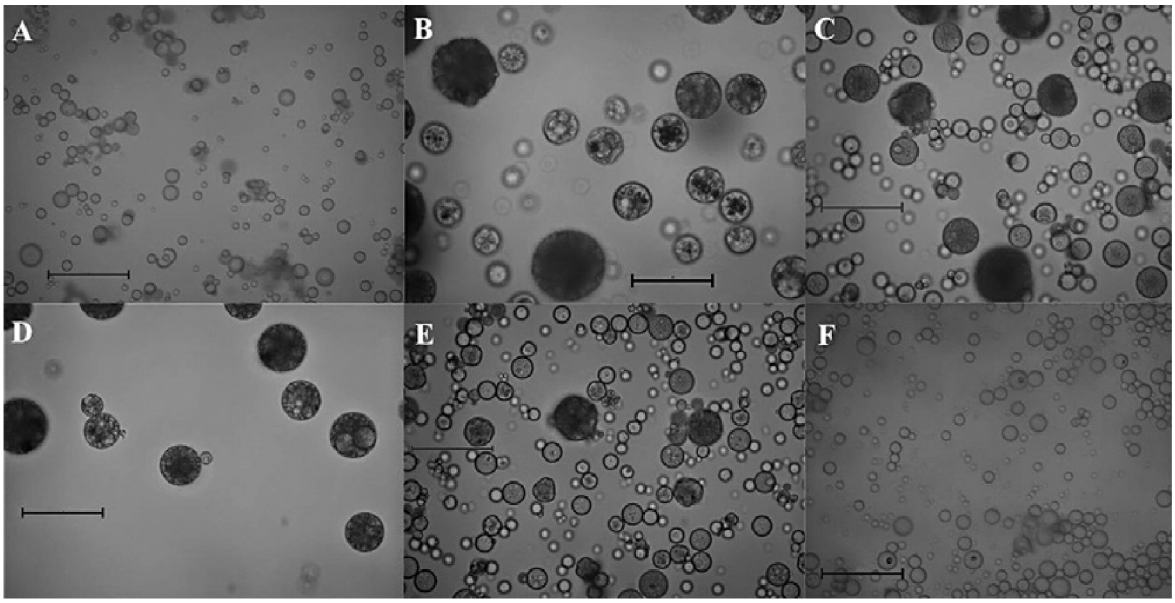

Figure 2. Optical microscope analysis – A) 753-G9 sample (bar = 50 µm); B) A504-G2.5 sample (bar = 50 µm); C) A653-$G9_1$ sample (bar = 50 µm); D) A653-$G9_{0.1}$ sample (bar = 50 µm); E) L653-G9 sample (bar = 50 µm); F) C753 sample (bar = 50 µm).

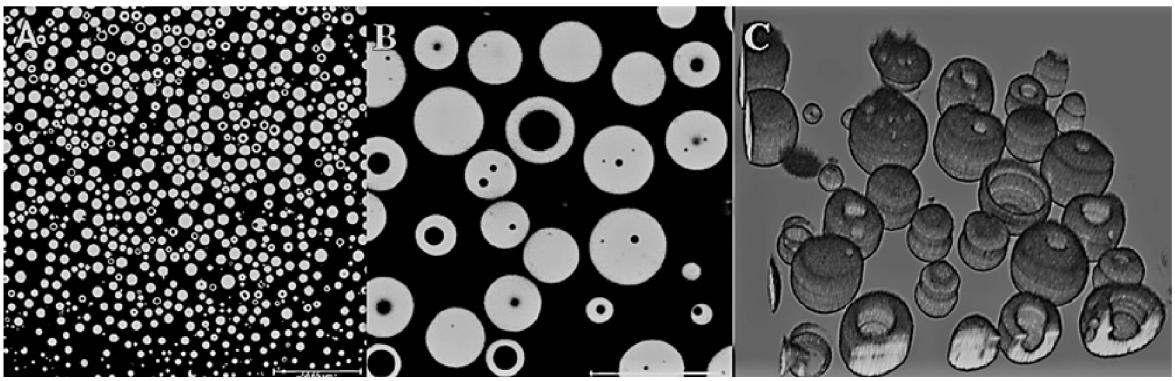

Figure 3. Confocal microscopy analysis of sample C753: A) bar = 56.65 µm; B) bar = 14.62 µm; C) 3D projection.

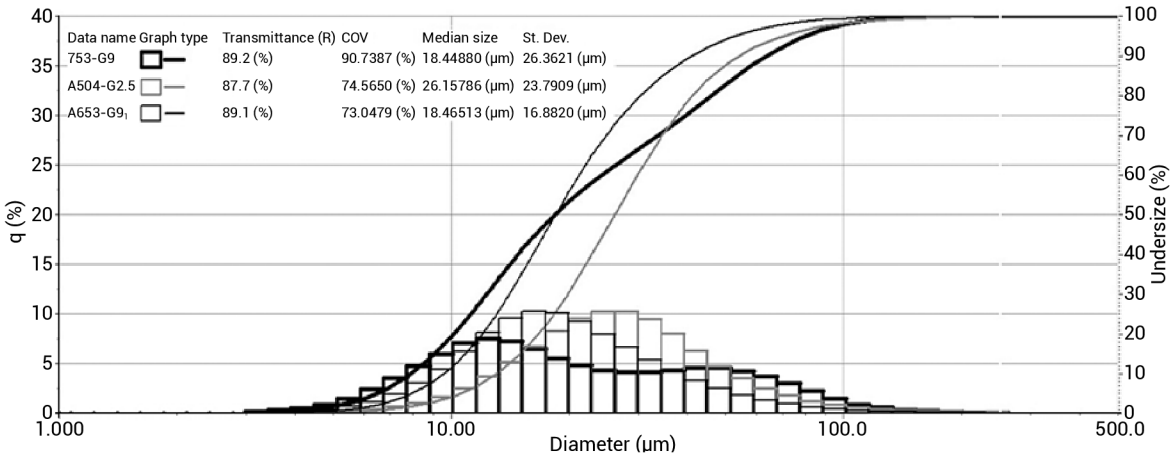

Figure 4. Laser diffraction of samples 753-G9, A504-G2.5, and A653-G9$_1$.

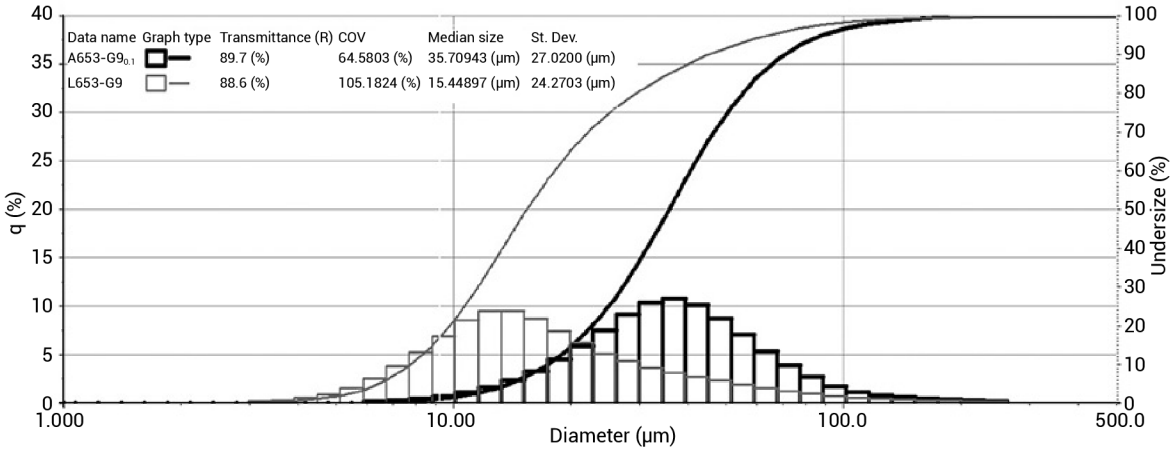

Figure 5. Laser diffraction of samples A653-G9$_{0.1}$ and L653-G9.

measured by laser diffraction is higher. Since microscope images clearly show particle size, the diffraction results suggest the presence of microparticle agglomerates, previously visible in SEM analysis results. They could be caused by imperfect resuspension in water. A solution could be seen in using a surfactant for better wetting (39, 40). Therefore, a medium for eventual *in vivo* administration should contain a surfactant to facilitate injectability. A surfactant should be selected based on its properties, interaction potential, and toxicological profile to minimize potential adverse effects (41).

In vitro release studies

GnRH analogs and metoclopramide dissolution test

A dissolution test is a form of evaluation, which, among other things, helps estimate behavior in terms of drug release. Figure 6 shows that almost the whole alarelin amount was released during the first hours of the Franz cells dissolution test. The sample A653-G9$_1$ released a significantly smaller drug amount than the A504-G2.5 sample. It could be attributed mainly to the 9.09% gelatine solution used as an inner aqueous phase thickener. When

Table 3. Laser diffraction measurement of microparticles particle size.

Sample	Median (µm)	Diameter (µm)	SD (µm)	D10 (%)	D50 (%)	D90 (%)
753-G9	18.4	29.1	26.4	7.9	18.4	64.2
A504-G2.5	26.2	31.9	23.8	13.2	26.2	54.7
A653-G9$_1$	18.5	23.1	16.9	9.6	18.5	41.4
A653-G9$_{0.1}$	35.7	41.8	27.0	17.6	35.7	71.1
L653-G9	15.4	23.1	24.3	7.8	15.4	45.6

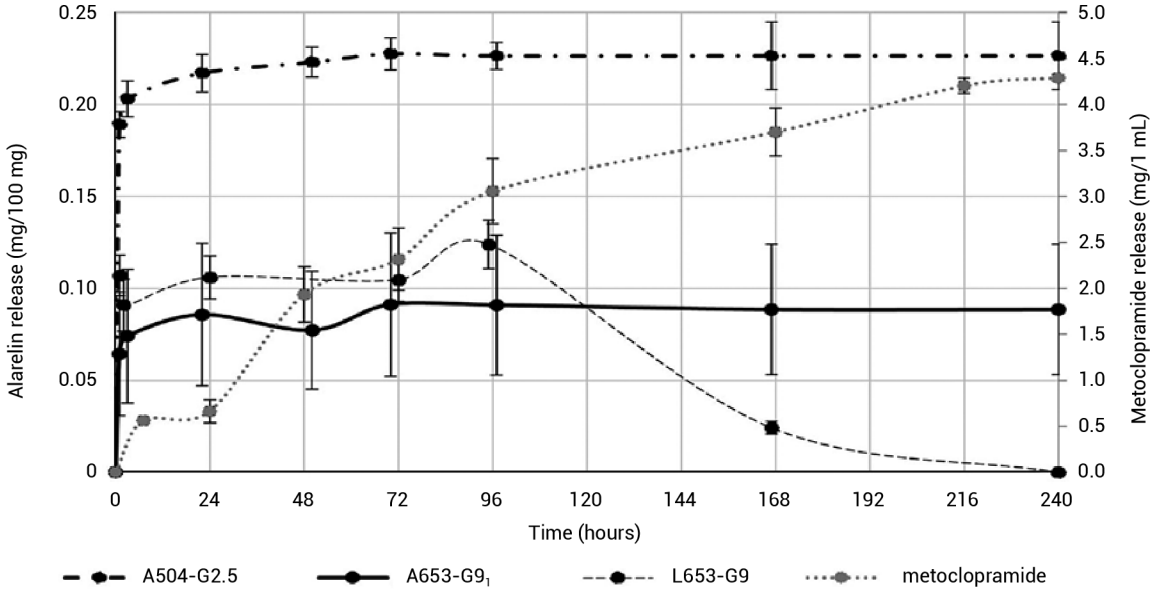

Figure 6. Dissolution profiles of A504-G2.5, A653-G9$_1$, L653-G9, and metoclopramide sample (5 ± 0.5°C; phosphate buffer - pH 7, 20-30 mL).

solidified, it is a very rigid thermoreversible gel. Only the outer layer of a gelatine microparticle core was hydrated after contact with an aqueous medium. It could be a reason for such limited drug release compared to the 2.5% gel used in the corresponding sample. This finding can be supported by experimental data published by Yu et al. In this study, the 5% gelatine gel of the inner water phase ensured a sustained release of highly soluble losartan potassium. Although the molecular weight of this drug is significantly lower (Mw 461.0) than that of alarelin (Mw 1167.3), a prolonged-release for 30 days has been observed (42). Figure 6 also shows that leuprorelin lost its stability over time and was decomposed in an aqueous medium. Thus, this sample was discarded for subsequent dissolution tests.

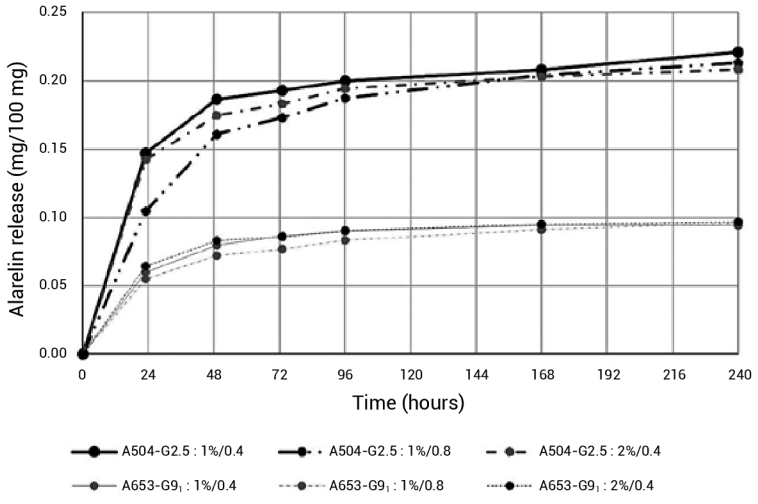

Figure 7. Release profiles of the alarelin samples A504-G2.5 and A653-G9$_1$ in agar gels differing in concentration of agarose and its volume (A504-G2.5: 1%/0.4 SD_{max} = 0.022; A504-G2.5: 1%/0.8 SD_{max} = 0.006; A504-G2.5: 2%/0.4 SD_{max} = 0.013; A653-G9$_1$: 1%/0.4 SD_{max} = 0.013; A653-G9$_1$: 1%/0.8 SD_{max} = 0.015; A653-G9$_1$: 2%/0.4 SD_{max} = 0.004 mg/100 mg – unit for all SD_{max}).

Due to the drug's immediate release during the dissolution test in buffer solutions, it was decided that a model imitating parenteral tissue administration is needed to obtain a more accurate preview. The previously suggested gel dissolution (25) was optimized and adjusted for the current experiment. Figure 7 shows that the drug dissolution in gels was considerably slower with effective drug release at least in the interval of 48-72 hrs. The difference between samples in terms of gelatine concentration has been preserved, as A504-G2.5 released twice the amount of A653-G9$_1$. The various adjustments to gel layers did not significantly influence the drug release. The only mild difference can be seen in sample A504-G2.5 in 0.8 mL/1% agarose gel, as this dissolution profile is slightly slower than its counterparts. It is for further investigation to decide the effect of polymer selection and gelatine concentration on drug release. However, the results showed potential for drug release profile modulation, which could be helpful when treating different fish species with varying reproduction requirements. Not to mention

the more samples can be easily combined in one dose.

The dissolution behavior of the metoclopramide suspension was investigated using a non-compendial exploratory biphasic dissolution method. It is a possible approach to assess the concurrent *in vivo* drug absorption process, and the used organic phase (octanol) simulates the transition through lipophilic cell membranes (43). The dissolution profile of the metoclopramide suspension (Figure 6) indicates that the drug gradually merged into an organic phase within ten days. Metoclopramide is a dopamine receptor antagonist, which can be used as an adjuvant during the GnRHa treatment. It increases therapy efficacy by blocking endogenous dopamine inhibition of the GnRH axis. In combination with the alarelin-loaded microparticles, this dissolution profile could be suitable for improving the reproduction of commercially farmed fish species. The obtained profile also gives a better insight into its behavior in tissues with more liquid content, like peritoneum, which can be beneficial when deciding the dose.

Resuspension index

Resuspension index (RI) was determined to assess the reproducibility of a drug dosing during a longer time interval when a microparticle suspension from one batch/package is used to treat multiple humans or animals at the same time. The higher RI corresponds to the slower particle sedimentation and the drug's exact dosing without repeated resuspending. The RI can be evaluated at different time intervals (44, 45), depending on the internal requirements and expected duration of specimen administration. The presented study assessed the RI for the drug-free 753-G9 microparticles exhibiting a comparable particle size to the alarelin-loaded sample. The obtained value was 70.99 ± 6.47%, meaning most of the particles remained resuspended, and the suspension is usable. Nevertheless, brief vortexing in regular intervals would be desirable for reproducible doses during fish population inoculation.

Pierce chromogenic endotoxin test

Figure 8 shows that all tested samples were contaminated by endotoxins to various degrees (0.29–1.66 IU/mL). Pierce chromogenic endotoxin test revealed that the o/w method is less susceptible to lipopolysaccharide fragments (LPS) contamination than the $w_1/o/w_2$ technique. It points to gelatine as the most critical factor (46). Despite using gelatine suitable for microbiology and depyrogenation protocols, it wasn't possible to prepare fully uncontaminated microparticles using any method. Thus, outside the preparation of experimental dosage form, it would be necessary to establish microparticles preparation under the cleanroom conditions to prevent LPS contamination. Also, unique methods of adjusting gelatine to decrease LPS content are available (47). However, it should be noted that unlike higher animals, which are extremely sensitive to endotoxins even at low doses, lower vertebrates like fish are often resistant to endotoxic shock (48).

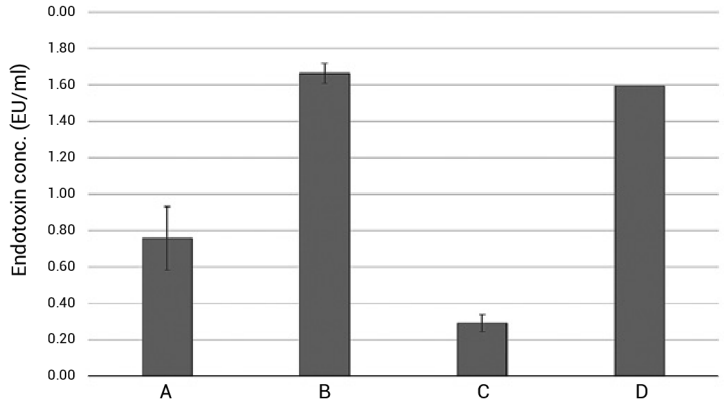

Figure 8. Pierce chromogenic endotoxin test - A) the sample without any drug technologically corresponding to the sample C753 (o/w technique) under non-pyrogenic conditions; B) the sample 753-G9 ($w_1/o/w_2$ technique) under non-pyrogenic conditions; C) the sample C753 (o/w technique) under non-pyrogenic conditions; D) the sample 753-G9 ($w_1/o/w_2$ technique) not under non-pyrogenic conditions.

CONCLUSION

A comprehensive approach to controlling aquaculture reproduction was suggested during the pre-formulation study. The PLGA microparticles prepared by the $w_1/o/w_2$ evaporation method were shown to be a potentially suitable carrier for the long-term release of GnRH analog alarelin. Their combination with metoclopramide suspension releasing the drug in a prolonged manner was demonstrated as a viable option. As a part of a microparticle core, the gelatine was revealed to be a key factor modifying *in vitro* drug release characteristics, as was demonstrated using dissolution test in agar medium mimicking *in vivo* conditions. A follow-up study was suggested to investigate polymer type/gelation concentration. Excellent characteristics, including high yield,

regular spherical shape, suitable particle size, and resuspension behavior, open the possibility of their trouble-free application by injection into a fish body. LPS contamination, originating probably from using gelatine solution, drew attention to the problem that would have to be solved in the case of broader use.

Acknowledgments

This experimental work was supported by the Ministry of Agriculture, Czech Republic, Project No. QK1810221 and the Ministry of Education, Youth and Sports of the Czech Republic, Project No. CZ.0 2.1.01/0.0/0.0/15_003/0000495.

Conflicts of interest

The authors declare no conflict of interest.

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CHAPTER 3

EFFICACY OF POLY (LACTIC-CO-GLYCOLIC ACID) MICROPARTICLES AS A GONADOTROPIN-RELEASING HORMONE ANALOGUE DELIVERY SYSTEM TO STIMULATE OVULATION OF PELED *COREGONUS PELED*

Knowles, J., Vysloužil, J., Muselík, J., Stejskal, V., Kouřil, J., Podhorec, P., 2021. Efficacy of poly (lactic-co-glycolic acid) microparticles as a gonadotropin-releasing hormone analogue delivery system to stimulate ovulation of peled *Coregonus peled*. Czech Journal of Animal Science 66, 331–338.

My share on this work was about 60%.

Original Paper *Czech Journal of Animal Science, 66, 2021 (08): 331–338*

https://doi.org/10.17221/57/2021-CJAS

Efficacy of poly (lactic-co-glycolic acid) microparticles as a gonadotropin-releasing hormone analogue delivery system to stimulate ovulation of peled *Coregonus peled*

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Citation: Knowles J., Vysloužil J., Muselík J., Stejskal V., Kouřil J., Podhorec P. (2021): Efficacy of poly (lactic-co-glycolic acid) microparticles as a gonadotropin-releasing hormone analogue delivery system to stimulate ovulation of peled *Coregonus peled*. Czech J. Anim. Sci., 66: 331–338.

Abstract: The aim of the study was to determine the efficacy of poly (lactic-co-glycolic acid) microparticles as a carrier of gonadotropin-releasing hormone analogues (GnRHa) for induction of ovulation in peled *Coregonus peled*. Female peled were injected intraperitoneally with 1) saline solution only (control), 2) mammalian GnRHa at 25 µg/kg body weight, 3) GnRHa in 753-type microparticles at 50 µg/kg, or 4) GnRHa in 653-type microparticles at 50 µg/kg. Blood plasma samples were taken on days 0, 4, 8, and 12 post-injection. All hormone treatments induced synchronous ovulation and higher cumulative ovulation compared to controls. Hormone treatments did not affect relative fecundity or the percentage of eyed eggs. Testosterone level decreased toward the onset of ovulation. On day eight of the trial, the testosterone level was significantly lower in hormone-treated groups compared to the control group. The level of 17β-oestradiol showed a decreasing trend post-injection, with the lowest observed level on day eight. Our results demonstrate that ovulation can be induced in the peled by the sustained – release of GnRHa in poly (lactic-co-glycolic acid) microparticles, but the treatment does not improve reproductive performance.

Keywords: induction of ovulation; GnRHa; salmonids; sustained drug release; steroid feedback

Peled *Coregonus peled* is native to Russia and has been introduced into areas including Belarus, Germany, Finland, Latvia, Estonia, and Czechia (Gordeeva et al. 2008). It has been successfully adapted to intensive aquaculture (Savini et al. 2010; Stejskal et al. 2018; Matousek et al. 2020). Peled matures at two years, with spawning typically occurring in winter. Broodstock must be regularly checked for ovulation during the reproductive season since spontaneous spawning can extend over several weeks. Temperature below 2 °C may stimulate mass ovulation (Hochman 1987).

Acceleration of sexual maturation and synchronisation of spawning offers the potential to increase the economic efficiency of gamete collection (Olito et al. 2001). Synthetic analogues of gonadotropin-re-

Supported by the Ministry of Agriculture of the Czech Republic – NAZV (Project No. QK1810221) and Grant Agency of the University of South Bohemia in Ceske Budejovice (GAJU 099/2019/Z).

Original Paper Czech Journal of Animal Science, 66, 2021 (08): 331–338

https://doi.org/10.17221/57/2021-CJAS

leasing hormone (GnRHa) can be used to stimulate and synchronise ovulation in salmonids (Mikolajczyk et al. 2008; Noori et al. 2010; Anderson et al. 2017). The prolonged luteinising hormone surge that is required for ovulation and spawning can be provided by multiple injections or by sustained drug release systems (Zohar and Mylonas 2001). Long-acting preparations show advantages over conventional administration, providing convenience, controlled release of the agent, low toxicity, and possibly greater effectiveness (Lin et al. 2014).

Administration in microparticles can optimise the effects of pharmaco-therapies. As a drug delivery system, microparticles offer protection of the active agent against enzymatic degradation, controlled drug release ranging from a few hours to several weeks, and easy administration not available with the standard long-acting preparations often used in aquaculture, such as implants (Siepmann and Siepmann 2006). Another advantage over implants is the simplicity of different release profiles that can be achieved by adjusting polymer selection, particle size and drug loading, and most importantly, the multiply character, which allows more homogenous distribution in the physiological environment (Vyslouzil et al. 2013). A wide range of biodegradable polymers has been used to formulate microparticles (da Silva and Pinto 2019; Shah et al. 2019; Strobel et al. 2020). Poly (lactic-co-glycolic acid) (PLGA) shows excellent biocompatibility and biodegradability in the body (Makadia and Siegel 2011), and its use in microparticles is a promising approach to induction of gametogenesis, showing high potential as a vehicle for long-acting pharmaceutical preparation in fish reproduction (Matejkova and Podhorec 2019).

The objectives of the study were to determine the efficacy of sustained GnRHa release from two types of PLGA microparticles in inducing ovulation in the peled, as well as its impact on egg quality and steroid feedback.

MATERIAL AND METHODS

The experiment was carried out in mid-December 2018 at a commercial fish farm in South Bohemia. On December 4, 2018, 250 sexually mature 3-year-old peled were obtained (Mydlovary, Czechia) from Kinski JSC Fish Farm (Zdar nad Sazavou, Czechia). Fish were sorted by sex and placed in separate flow-through tanks (four tanks for females, one for males) supplied with water from a local river and held for acclimatisation for seven days under ambient water temperature and photoperiod. Fish were not fed during the study. Water temperature decreased continuously from 7.2 °C at the beginning of the experiment to 3.5 °C during the incubation of eggs (Figures 1 and 2).

On December 11, females were randomly divided into four groups of 50 fish. Fish were anaesthetised in a clove oil bath (0.03 ml/l), and the preparations were injected intraperitoneally using a 2 ml syringe equipped with a 0.33 × 12 mm needle. The experimental groups were: CON: saline solution only (Braun Melsugen AG, Melsungen, Germany), a single injection of 0.9% NaCl at dose 1 ml/kg body weight; mGnRHa: single injection of Supergestran® (Nordic Pharma, Jesenice, Czech Republic) at 25 µg/kg body weight [mammalian GnRHa (D-Tle^6, Pro^9, NEt-mGnRHa)]; M753: 10-day release microparticles with encapsulated GnRHa [(D-Ala^6, des-Gly^{10}) GnRH-ethylamide] formulated with

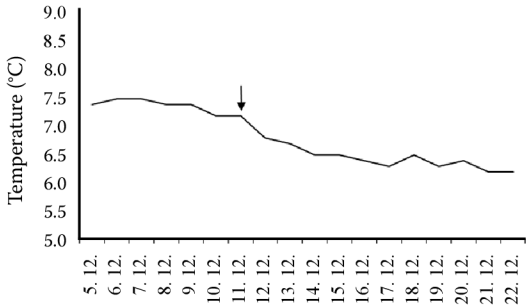

Figure 1. Water temperature in the fish tanks during the trial during December 2018
Arrow indicates the date of hormone injection

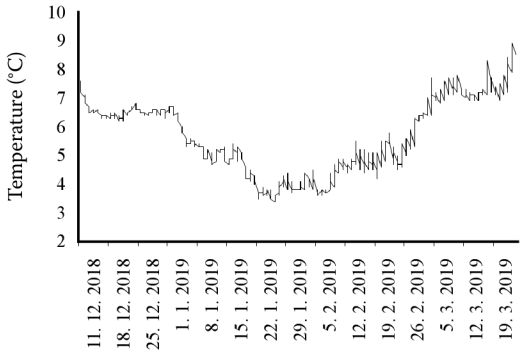

Figure 2. Water temperature during egg incubation of *Coregonus peled*

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Resomer RG 753H (Evonik, Darmstadt, Germany) at 50 µg/kg GnRHa with 5 µg/kg released per day; M653: 10-day release microparticles with encapsulated GnRHa, formulated with Resomer RG 653H (Evonik, Darmstadt, Germany), at 50 µg/kg GnRHa with 5 µg/kg released per day.

Resomer RG 753H that was used for the formation of PLGA applied in group M753 contains 10% more polylactic acid than Resomer RG 653H used in group M653, which decreases its hydrophilicity, suggesting that its use will provide slower onset of hormone release and a more prolonged effect.

After injection, fish were checked for ovulation at four-day intervals. Ovulation was considered to have occurred if eggs were released by a gentle massage of the abdomen. Ovulated eggs were stripped into separate dishes for each female, and a small sieve and filter paper were used to remove the ovarian fluid. Eggs from each female were weighed to 0.01 g (PCB 1000-2; Kern & Sohn GmbH, Balingen, Germany). A sample of unfertilised eggs was used to calculate the absolute and relative fecundity.

Sperm from 50 males that spontaneously released sperm upon the gentle massage of the abdomen was separately collected at the time of fertilisation into a 2 ml syringe. Eggs from each female were separately fertilised with an equal volume of sperm from three males. Sperm was mixed with the eggs, hatchery water was added, and the mixture was gently stirred for 1 h using a horizontal shaker (Duomax 1030; Heidolph Instruments GmbH, Schwabach, Germany). Three samples from each ovulating female, each containing ~150 eggs, were separately incubated in small plexiglass incubators (1.7 l), equipped with dirigible inflow (Kallert 2009) with regulated water flow. The mean pH was 7.62 ± 0.05, and oxygen saturation was maintained at 10.4 ± 1.06 mg/l. Water temperature was measured hourly by an automatic datalogger (EL-USB-1-RCG; Lascar Electronics, Whiteparish, UK). Dead eggs were counted and removed using a 3 ml plastic pipette. On February 5, the percent of eyed eggs in the total number of eggs in the incubator was calculated.

Microparticle preparation

The standard double water-in-oil-in-water ($w_1/o/w_2$) principle was used to prepare both microparticle treatments. Alarelin acetate (APExBIO, Houston, TX, USA) (10 mg) was dissolved in 1.5 g of 9.1% gelatine solution ($water_1$ phase) at 50 °C. Two types of Resomer with carboxyl groups on the polymer ends and different ratios of glycolic acid to lactic acid (65 : 35 for RG 653H and 75 : 25 for RG 753H) were used to investigate the optimal carrier properties for GnRHa encapsulation. Resomer was chosen since it has been found useful in various products (Schwach et al. 2003; Duvvuri et al. 2005)

For type 653 microparticles, 800 mg of Resomer RG 653H (0.32–0.44 dl/g) (Evonik, Darmstadt, Germany) was used, and 800 mg Resomer RG 753H (0.32–0.44 dl/g) (Evonik, Darmstadt, Germany) was used for type 753. Resomer was dissolved in 5 ml dichloromethane) (Penta, Prague, Czech Republic), pre-mixed on a vortex for 30 s, and homogenized (Ultra-Turrax T25; Ika Werke, Staufen Im Bresgau, Germany). The resulting w_1/o emulsion was pre-mixed for the 60 s on a homogeniser with 12 g of 1% poly (vinyl alcohol) solution at 50 °C (PVA, Mw 31 000–50 000; 98–99% hydrolysed; Sigma Aldrich, St. Louis, MO, USA) to create a concentrated double emulsion $w_1/o/w_2$. The concentrated emulsion was immediately poured into 200 g of 0.1% PVA/2.0% NaCl. The resultant $w_1/o/w_2$ emulsion was stirred for 2 h to completely evaporate dichloromethane. The particles were collected via centrifugation, resuspended in purified water, and lyophilised. Four runs were prepared from each Resomer.

Blood sampling and steroid assay

Blood samples were taken prior to the injection of the preparations (day 0), and on days 4, 8, and 12 post-injection. Ten females from each group were sampled at each sampling point and each female was sampled only once. Blood samples (1 ml) were taken by caudal venipuncture using heparinised syringes (5 ml) and 0.7 × 30 mm needles. Plasma was obtained by 10 min centrifugation at 5 000 RPM, 4 °C (5427R; Eppendorf AG, Hamburg, Germany) and stored at −80 °C until analysis. The level of sex steroids in the blood plasma were measured twice in each sample.

The concentration of testosterone (T) (KAPD-1559) and17β-oestradiol (E2) (KAP0621) were analysed by enzyme-linked immunosorbent assay using commercially available kits (DIAsource,

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Ottignies-Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. The samples were checked for dilution accuracy and diluted (1 : 2) with Calibrator 0, if necessary. The absorbance was read at 450 nm with a microplate plate reader (PlateReader AF2200; Eppendorf AG, Hamburg, Germany). The sensitivity of the assays was 0.083 ng/ml for T and 5 pg/ml for E2. Intra-assay coefficients of variation for all assays were less than 9%, and inter-assay coefficients of variation were less than 8%.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted with Statistica v12 Cz (StatSoft, Tulsa, OK, USA). Normality and homogeneity (Cochran C., Hartley, Bartlett) of data were tested and significant differences were analysed by one way ANOVA (Tukey or HSD test). For all tests, the level of significance was set at $P < 0.05$.

RESULTS

All treatments were associated with significantly greater synchronisation of ovulation than seen in the control group, with no significant differences found among the treated groups. First ovulation occurred four days post-injection in all hormone-treated groups, while first ovulation in the control group was recorded on day 12 of the experiment. On day 12, the percentage of ovulated females was 82.5% in mGnRHa, 70% in M753, and 90% in M653 (Figure 3). The latency period was significantly shorter in mGnRHa (8.4 ± 0.6 days) than in control (12 days), with no significant differences in latency found among the treated groups.

No significant differences were observed among groups in terms of relative and absolute fecundity. Mean relative fecundity was 19 087 ± 1 136 eggs/kg fish body weight (Figure 4).

The level of T decreased towards ovulation. The decrease was slighter in the controls compared to treatment groups. On day eight, the T level was significantly lower in treatment groups compared to controls, with no significant differences among treatments. At the end of the trial, plasma T levels were similar among groups, with no significant differences on day 12 (Figure 5).

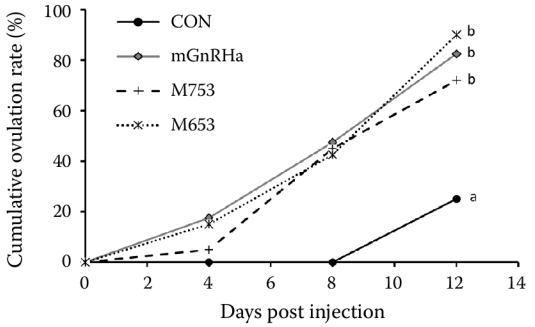

Figure 3. Cumulative ovulation rate of *Coregonus peled*
CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight
[a,b]Groups with different letters are significantly different ($P < 0.05$)

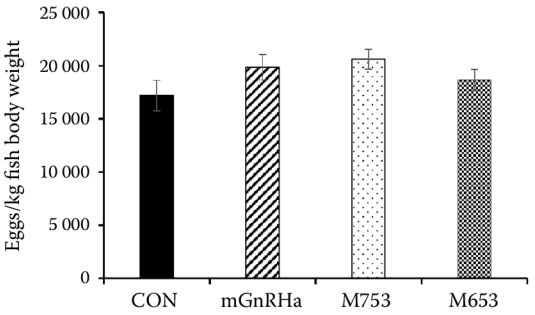

Figure 4. Relative fecundity in *Coregonus peled* in different treatments
CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

The E2 level showed a decreasing trend with the minimum level in hormone-treated groups on day eight and, in the control group, on day 12. The M753 group showed significantly lower E2 concentration on day eight compared to controls and mGnRHa. At the end of the trial, both microparticle-treated groups showed an increasing trend in E2 compared to controls (Figure 6).

There was no significant difference in the percentage of eyed eggs in samples from tested groups: CON 50.6 ± 1.6%; mGnRHa, 49.9 ± 1.3%; M753, 52.6 ± 1.2%; and M653, 54.1 ± 1.1%.

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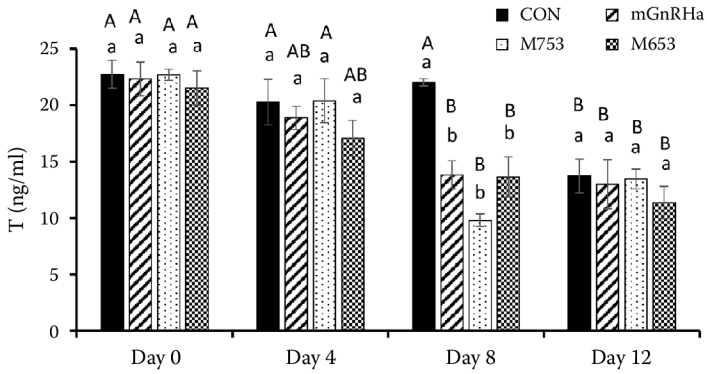

Figure 5. Changes in plasma testosterone (T) in *Coregonus peled*

CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

[A,B]Different letters indicate significant differences within an experimental group over the course of the experiment (one way ANOVA)

[a,b]Different letters indicate significant differences among groups on a sampling day (one way ANOVA)

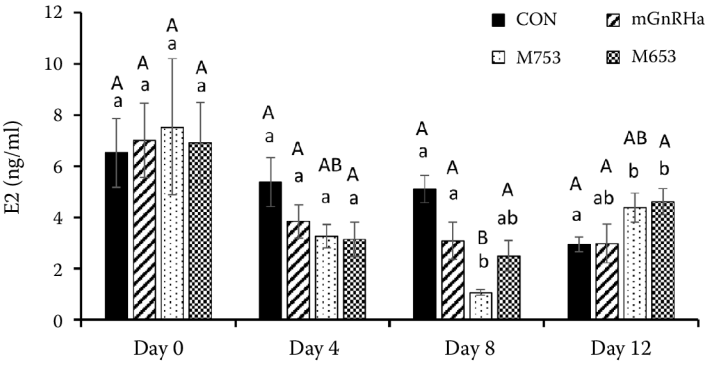

Figure 6. Changes in plasma 17β-oestradiol (E2) in *Coregonus peled*

CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

[A,B]Different letters indicate significant differences within an experimental group over the course of the experiment (one way ANOVA)

[a,b]Different letters indicate significant differences among groups on a sampling day (one way ANOVA)

DISCUSSION

Induction and synchronisation of ovulation in peled by single injection or prolonged-release GnRHa was significantly increased compared to non-hormone-treated controls. Similar results have been reported for other salmonids (Olito et al. 2001; Noori et al. 2010; Svinger et al. 2013). We found no difference in results of prolonged-release and acute treatment in peled, although a positive effect of prolonged released preparations compared to the acute administration has been frequently reported. Sustained-release GnRHa with Freund's incomplete adjuvant at 25–50 µg/kg in rainbow trout *Oncorhynchus mykiss* resulted in significantly higher ovulation compared to the acute hormone treatment (Arabaci et al. 2004; Vazirzadeh et al. 2008). In the same species, 100% ovulation was obtained with sustained-release GnRHa (12.5–50.0 µg/kg) encapsulated in a polyglycolic-

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polylactic matrix, results not reached with a single injection of GnRHa at 20 µg/kg (Breton et al. 1990). In the present study, a single injection of mGnRHa at 25 µg/kg was effective at induction and synchronisation of ovulation, in accordance with other studies in other salmonids (Mikolajczyk et al. 2007; 2008).

The hormone treatment in our experiment did not affect the relative fecundity or the proportion of eyed eggs. No effect of hormone treatment on the volume of eggs was observed in Atlantic salmon *Salmo salar* (Anderson et al. 2017). In some species, ovulation induced by GnRHa has been associated with a negative effect on egg quality (Bobe and Labbe 2010). GnRHa implants were associated with reduced egg buoyancy, fertilisation, number of viable eggs, and smaller oil globules (Agulleiro et al. 2006; Bonnet et al. 2007; Garber et al. 2009). On the other hand, long-lasting GnRHa implants in greater amberjack *Seriola dumerili* led to higher fecundity compared to spontaneous spawning and to single hormonal injections (Jerez et al. 2018). A positive effect of prolonged GnRHa release from implants was also observed in Atlantic salmon males in which total expressible milt increased, while sperm density and motility was unchanged (Goren 1995).

In our experiment, the percentage of eyed eggs was similar in all tested groups. A lack of effect of hormone treatment on egg quality was also reported by other authors (Billard et al. 1984; Dabrowski et al. 1994; Park et al. 2007; Zarski et al. 2019). In contrast, a lower percentage of eyed eggs was observed in brown trout *Salmo trutta* treated with a double injection of GnRHa at 10 µg/kg (Mylonas et al. 1992). Reduced number of offspring was reported in chinook salmon *Oncorhynchus tshawytscha* treated with GnRHa at 25 µg/kg compared untreated controls (Olito et al. 2001).

In the present study, the level of E2 and T showed a similar pattern in all groups, with levels decreasing near ovulation. However, T tended to decrease later than E2 in all groups. The trend was also observed in Atlantic salmon (Dickhoff et al. 1989; Taranger et al. 1998) and European grayling *Thymallus thymallus* (Szmyt et al. 2021). Similar results were observed in wild brown trout, in which the highest concentration of plasma E2 occurred 30 days prior to ovulation, while, in cultured brown trout, the peak level was 14 days before ovulation (Norberg et al. 1989).

CONCLUSIONS

Sustained delivery system and a single GnRHa injection can be successful in induction and synchronisation of ovulation in peled if administered near the natural spawning period. Poly (lactic-co-glycolic acid microparticle treatment does not increase fecundity or percentage of eyed eggs. Further investigation is needed to determine the effect of microparticles on the brood fish and the survival of the fry.

Conflict of interest

The authors declare no conflict of interest.

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Received: April 6, 2021
Accepted: May 22, 2021
Publish online: July 27, 2021

CHAPTER 4

SPAWNING PERFORMANCE AND SEX STEROID LEVELS IN FEMALE PIKEPERCH *SANDER LUCIOPERCA* TREATED WITH POLY(LACTIC-CO-GLYCOLIC ACID) MICROPARTICLES

Knowles, J., Vysloužil, J., Policar, T., Milla, S., Holická, M., Podhorec, P., 2022. Spawning Performance and sex steroid levels in female pikeperch *Sander lucioperca* treated with poly(lactic-co-glycolic acid) microparticles. Animals 12, 208.

My share on this work was about 50%

animals

MDPI

Article

Spawning Performance and Sex Steroid Levels in Female Pikeperch *Sander lucioperca* Treated with Poly(lactic-co-glycolic acid) Microparticles

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Simple Summary: Pikeperch *Sander lucioperca* is a promising candidate for intensive aquaculture. However, controlled reproduction has become the major bottleneck in pikeperch production. To improve and optimize its artificial reproduction, an effective method for hormone treatment is needed. The use of a poly(lactic-co-glycolic acid) microparticle-sustained-release system to administer gonadotropin-releasing hormone agonist to pikeperch resulted in acceptable reproductive output. Our results establish the potential of poly(lactic-co-glycolic acid) microparticle as a novel tool for hormone treatment in fish.

Abstract: Pikeperch *Sander lucioperca* is a piscivorous species considered a promising candidate for the diversification of intensive aquaculture. This study aimed to determine the effect of a sustained-release delivery system incorporating mammalian gonadotropin-releasing hormone agonist (mGnRHa) into poly(lactic-co-glycolic acid) (PLGA) microparticles on the sex steroid levels and aspects of artificial reproduction of pikeperch. Fish were divided into four groups and injected with 20 µg mGnRHa/kg, 5-day release microparticles encapsulated with 5 µg GnRHa/kg BW (PLGA 5), 20 µg GnRHa/kg (PLGA 20), or 1 mL/kg 0.9% NaCl (control). Cumulative percentage ovulation was 100% in the PLGA 5 group, significantly higher than in other tested groups. No differences among groups were observed in latency or fecundity. The level of 11-ketotestosterone (11-KT) peaked at 40 h post-injection, and was sustained during ovulation, in all treated groups. The 17β-estradiol (E2) concentration increased in the mGnRHa-only group immediately after hormone injection, while both PLGA groups showed a reduction in E2 after injection, continuing to decrease until ovulation. A low dose of mGnRHa in PLGA microparticles significantly improves induction of ovulation and results in acceptable reproductive performance, which may positively affect pikeperch production under controlled conditions.

Keywords: aquaculture; GnRHa; induced ovulation; reproductive dysfunction; sustained drug release

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Citation: Knowles, J.; Vysloužil, J.; Policar, T.; Milla, S.; Holická, M.; Podhorec, P. Spawning Performance and Sex Steroid Levels in Female Pikeperch *Sander lucioperca* Treated with Poly(lactic-co-glycolic acid) Microparticles. *Animals* **2022**, *12*, 208. https://doi.org/10.3390/ani12020208

Academic Editor: Tien-Chieh Hung

Received: 18 November 2021
Accepted: 13 January 2022
Published: 16 January 2022

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1. Introduction

Pikeperch *Sander lucioperca* is the most valuable freshwater fish species for European intensive aquaculture due to its exquisite meat quality, good growth performance, and high market demand [1]. Like other Percidae, pikeperch is an annual spawner with group synchronous ovarian development [2]. Spawning usually occurs from March through May when water temperature is 10–16 °C [3]. The controlled reproduction of pikeperch has long

challenged aquaculturists, with initial attempts to obtain gametes of the species reported in 1928 [4]. To date, the methods usually used to accomplish this are natural spawning [5] or support of spawning by placing artificial nests (synthetic turf, brushes, coconut mats) inside rearing tanks [6–8]. These methods have the advantages of minimal handling and labor, but are often associated with low fertilization and with loss of eggs due to spawning outside the nest [8,9]. The techniques also do not allow the controlled reproduction of individual fish, often required for breeding programs or triploidisation [10], necessitating stripping for in vitro fertilization. Although it shows advantages, artificial reproduction followed by in vitro fertilization is a challenging process complicated by difficulty in timing of stripping, spontaneous eggs release, post-ovulatory ageing, high broodstock mortality, and variable egg quality [8,11].

Fish artificial reproduction aided by hormone stimulation dates to the early 20th century. Hormonal induction of ovulation in fish involves use of either gonadotropins or a gonadotropin-releasing hormone analogue (GnRHa) in a liquid or sustained-release delivery system [12]. In pikeperch, the commonly used preparations for induction of ovulation are human chorionic gonadotropin (hCG) and GnRHa [8,9]. In pikeperch, hCG treatment has a negative effect on egg quality [11] and can enhance cortisol production [13] that may further impact egg quality [14].

In addition to the hormone used, the method of administration is an important factor influencing treatment efficacy [15]. The development of an advanced drug delivery system opens new possibilities for avoiding multiple injections and enabling the release of bound hormones at a desired dose for a predetermined period of time [16]. In the past 20 years, a variety of sustained GnRHa delivery systems have been successfully tested in induction of ovulation and spermiation in fish. Among them, copolymer of ethylene [17] and cholesterol pellets [18] have been the most used in induction of reproduction in fish. Nevertheless, it is important to highlight that these delivery systems are used to induce ovulation and spermiation particularly in marine fish and salmonids [17]. Several more sustained drug delivery systems, such as Freund's incomplete adjuvant [19], microspheres of copolymer of fatty acid dimer, sebacic acid, lactide-glycolide [20], and chitosan-gold nanoconjugates [21], have been tested in fish. However, these systems did not gain wider commercial use in the treatment of freshwater fish reproductive dysfunctions. Among drug delivery systems used in veterinary and human medicine, the poly(lactic-co-glycolic acid) (PLGA) microparticles have emerged as one of the most promising matrixes for binding drugs, due to its simple preparation [22], biocompatibility, biodegradability [23], and its facility of encapsulating a wide variety of active substance [24–26].

The aim of this study was to determine the effect of hormone treatment using mammalian gonadotropin-releasing hormone agonist (mGnRHa) incorporated into PLGA microparticles in pikeperch females and compare its efficacy with that of mGnRHa treatment alone and with physiological saline solution.

2. Materials and Methods

2.1. Experimental Groups and Design

Pikeperch females (n = 40; 818 ± 295 g) were collected from earthen ponds in South Czechia in late March and transported to the experimental facility of the Faculty of Fisheries and Protection of Waters in Vodnany (49°N, 14°E) where they were held in storage ponds and fed forage fish *Pseudorasbora parva* until the spawning season in April. Oocyte maturity stage was determined in each female according to described methods [10]. Oocytes were collected from the urogenital papilla via a polyethylene cannula (2.7 mm) and the sampled oocytes were treated with a clearing solution (ethanol:formalin:glacial acetic acid in the ratio of 6:3:1). Females at oocyte maturation stage III were randomly divided into four groups of 10 and intramuscularly injected under the dorsal fin according to the following protocols:

- 0.9% NaCl: saline solution only (Braun Melsungen AG, Melsungen, Germany), single injection of 0.9% NaCl at 1 mL/kg body weight (BW).

- mGnRHa: single injection of Supergestran® (Nordic Pharma, Jesenice, Czech Republic) at 20 μg/kg BW [mGnRHa (D-Tle^6, Pro^9, NEt-mGnRHa)].
- PLGA 5: 5-day release microparticles with encapsulated GnRHa ([D-Ala^6, des-Gly^{10}] GnRH-ethylamide) (APExBIO, Houston, TX, USA) at 5 μg/kg BW = GnRHa at 1 μg/kg BW per day.
- PLGA 20: 5-day release microparticles with encapsulated GnRHa ([D-Ala^6, des-Gly^{10}]GnRH-ethylamide) (APExBIO, Houston, TX, USA) at 20 μg/kg BW GnRHa = 4 μg/kg BW per day.

The choice of doses used in the present study were based on the findings of various studies. A single injection of dose 5 μg GnRHa/kg BW have not proven being successful in formerly published studies and thus this group was not included in the experiment [27,28]. Following injection, each group was kept in a 1 m^3 flow-through tank under ambient water temperature. Water temperature was measured hourly by an auto-recording data logger (EL-USB-1-RCG, Lascar Electronics, Whiteparish, UK) (Figure 1). The oxygen saturation (%) and pH were measured daily at 06:30 and 14:00 h with a combined pH and oxygen meter (MultiLine P4, WTW, Weilheim, Germany). Water quality parameters were pH 7.2 ± 0.2, oxygen saturation 80.4 ± 9.1%, flow rate 12.5 L/min, temperature 15.1 ± 0.96 °C, ammonia concentration, <0.02 mg/L; nitrite, <0.02 mg/L; and photoperiod 14L:10D with light intensity of 30 lx.

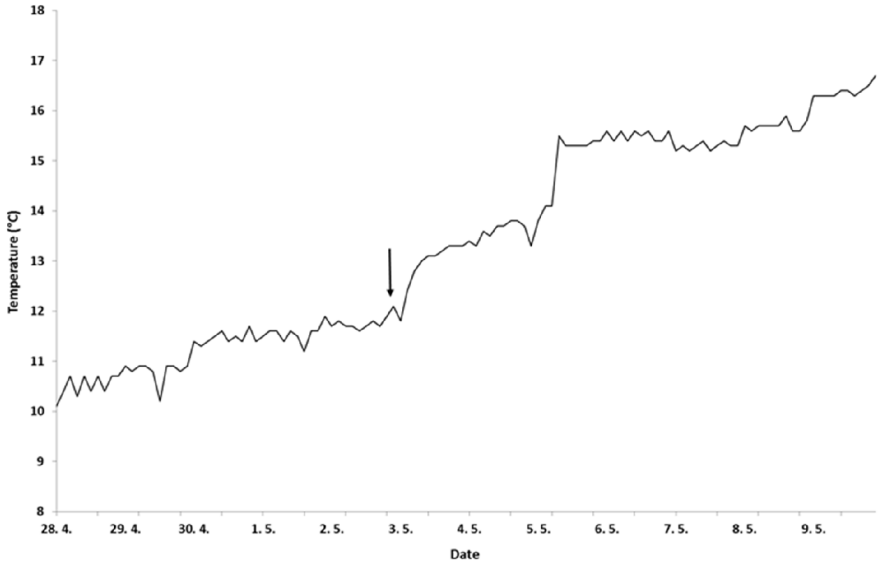

Figure 1. Water temperature over the course of 12 days auto-recorded by data logger (Lascar Electronics, EL-USB-1-RCG) at 1-h intervals. Arrow indicates the date of hormone injection.

Blood (2 mL) was collected by caudal venipuncture into 5 mL heparinized syringes from each fish at 0, 20, 40, and 168 h post-stimulation for steroid level analysis. Plasma samples were obtained by centrifugation at 1500× *g* for 10 min at 10 °C (Eppendorf 5427 R, Eppendorf, Hamburg, Germany) and immediately frozen and stored at −80 °C until analyses.

2.2. Microparticle Formation

Microparticle preparation used a standard water-in-oil-in-water combination (w1/o/w2). Ten mg of alarelin acetate (APExBIO, Houston, TX, USA) was dissolved in 1.5 g of warmed (50 °C) 9.1% gelatine solution (w1). PLGA RESOMER® RG 753H (800 mg) (Evonik, Darmstadt, Germany) was dissolved in 5 mL dichloromethane (Penta, Prague, Czech Republic) (oil phase). The w1 and oil phases were pre-mixed by vortexing for 30 s and homogenized for 60 s (Ultra-Turrax T25, Ika Werke, Staufen Im Bresgau, Germany). The resulting

w1/o emulsion was pre-mixed for 60 s on a homogenizer with 12 g of warmed (50 °C) 1% poly(vinyl alcohol) solution (PVA, Mw 31,000–50,000, 98–99% hydrolyzed; Sigma Aldrich, St. Louis, MO, USA) to create a concentrated double emulsion w1/o/w2. The concentrated emulsion was immediately poured into 200 g of 0.1% PVA/2.0% NaCl. The resulting w1/o/w2 emulsion was stirred for 2 h to completely evaporate dichloromethane. Particles were collected by centrifugation, re-suspended in purified water, and lyophilized.

To provide information about drug release from the PLGA microparticles, an in vitro dissolution test was carried out in 10 mL glass vials at 15.0 °C ± 0.5 °C using 100 mg PLGA microparticles with alarelin acetate embedded in 0.4 mL 1% agar gel. After the gel solidified, an additional 0.8 mL agar gel was applied as a cover layer. After solidification, 5 mL of pH 7.0 phosphate buffer was added. Samples were taken for seven days. The entire volume of dissolution medium was removed and replaced with fresh buffer. The dissolution was performed in triplicate. Determination of alarelin acetate in dissolution medium was performed by HPLC (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) on a Nucleodur 10-5 CN-RP column (Machery-Nagel, Duren, Germany) with acetonitrile: 20 mM H_3PO_4 (16:84 *v*/*v*) at a flow rate of 0.8 mL/min and spectrophotometric detection at 220 nm (Figure 2).

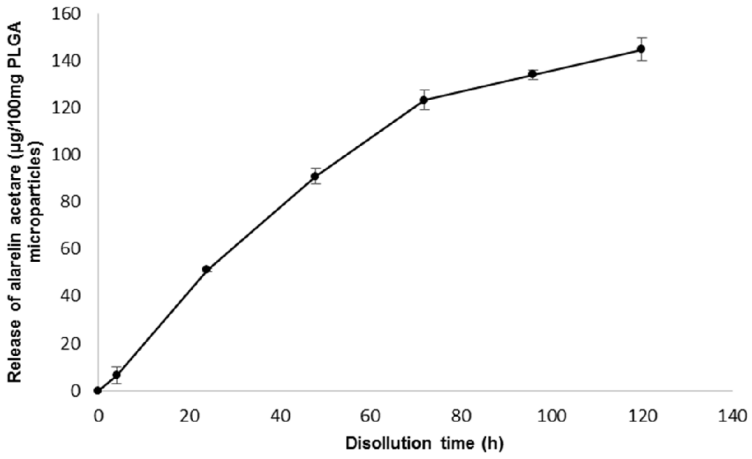

Figure 2. Dissolution profile of alarelin acetate release from poly(lactic-co-glycolic acid) microparticles.

2.3. Stripping of Broodfish

Beginning 48 h post-hormone treatment, the fish were examined hourly by a gentle abdominal massage. Those showing signs of ovulation were immediately transferred to a clove oil water bath (0.03 mL/L), and eggs were stripped by gentle abdominal massage under dry conditions into a dry bowl. Eggs were weighed using a balance (PCB 1000-2, Kern, Germany with accuracy of 0.01 g) separately for each female. Three samples of ~1 g per spawning were randomly selected and weighed using a balance (ALJ 220-4, Kern, Balingen, Germany) with accuracy of 0.0001 g and counted for determination of absolute fecundity (total number of eggs per female) and relative fecundity (total number of eggs per kg BW).

After fertilization according to a previously described method [28], three samples of ~100 eggs were counted and incubated in separate incubators in a recirculating system at 16 ± 0.5 °C as described by Blecha et al. [6]. Hatching began on the fifth day post-spawning and the freshly hatched free-swimming larvae were counted. The hatching rate was determined as follows:

$$\text{Hatching rate} = (\text{NL}/\text{NE}) \times 100 \quad (1)$$

where NL is the number of hatched larvae and NE is the total number of eggs stocked in the incubator.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma levels of testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) were quantified by ELISA, and each standard and plasma sample was run in duplicate.

Concentration of T (KAPD1559) and E2 (KAP0621) were analyzed by ELISA using commercially available kits (DIAsource, Ottignies-Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. The androgen 11-KT was assayed with the Cayman 11-KT EIA kit (582751, Cayman Chemical, Ann Arbour, MI USA). The intra-assay coefficients of variation (calculated from the duplicate sample) were less than 7% in all tests and inter-assay coefficients of variation were less than 6.5%. Absorbance was read with a microplate plate reader (PlateReader AF2200, Eppendorf, Hamburg, Germany).

2.5. Data Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted with Statistica v. 12 CZ (StatSoft, Tulsa, OK USA). Normality and homogeneity (Cochran C) of data were tested on raw data, percentage data were arcsin transformed, and steroid data log transformed to satisfy homogeneity of variance requirements. Significant differences were analyzed by one-way ANOVA. If significant differences were found by ANOVA, Tukey, or HSD test were applied for detailed multicomparison assay. Ovulation rate was analyzed by the $\chi 2$ analysis. For all tests, the level of significance was set at $p < 0.05$.

3. Results

Ovulation rate (number of ovulated females during the experiment) was 100% in the group treated with 5 μg GnRHa/kg BW (PLGA 5) and was significantly higher than rates obtained in other groups. Cumulative ovulation in groups treated with mGnRHa only and with PLGA 20 was 40%, while no ovulation was seen in the saline-only control group. The latency period (time from injection to ovulation) ranged from 80 to 128 h post-hormone treatment with no significant differences among treated groups. No significant differences were found among groups in relative or absolute fecundity, and high inter-individual variation was recorded. High SEM of both measures of fecundity reflected the wide range in egg numbers obtained from individual females. Hatching rate was 53.4–68.0% in the successfully spawning groups with no significant differences among groups (Table 1).

Table 1. Characteristics of ovulation in pikeperch relative to mGnRHa delivery protocol. Fish were injected with 0.9% NaCl at 1 mL/kg; single injection of mGnRHa at 20 μg/kg; 5 μg GnRHa/kg BW embedded in PLGA microparticles (PLGA 5); and 20 μg GnRHa/kg BW embedded in PLGA microparticles (PLGA 20).

Group	Injected/Ovulated	Latency Period (h)	Absolute Fecundity (Eggs/Female)	Relative Fecundity (eggs/kg BW)	Hatching Rate (%)
0.9% NaCl	10/0 [a]				
mGnRHa	10/4 [a]	84.5 ± 0.65	85,094 ± 39,731	83,943 ± 36,100	57.7 ± 7.9
PLGA 5	10/10 [b]	96.8 ± 1.03	109,781 ± 24,872	128,184 ± 27,811	68.0 ± 4.4
PLGA 20	10/4 [a]	90.5 ± 1.95	61,784 ± 41,289	52,784 ± 31,868	53.4 ± 5.1

[a,b] Different superscripts within a column indicate significant difference ($p < 0.05$).

The level of plasma T increased in the PLGA groups 20 h after hormone stimulation compared to the control group but declined precipitously at ovulation. 11-KT showed an increasing trend from 40 h post-hormone treatment in all groups with highest level at ovulation time. No significant effect was reported with either androgen. Plasma E2 level increased in the mGnRHa group immediately upon hormone administration compared

to the PLGA groups. However, such inter-group difference was not detectable after 40 h (Figure 3).

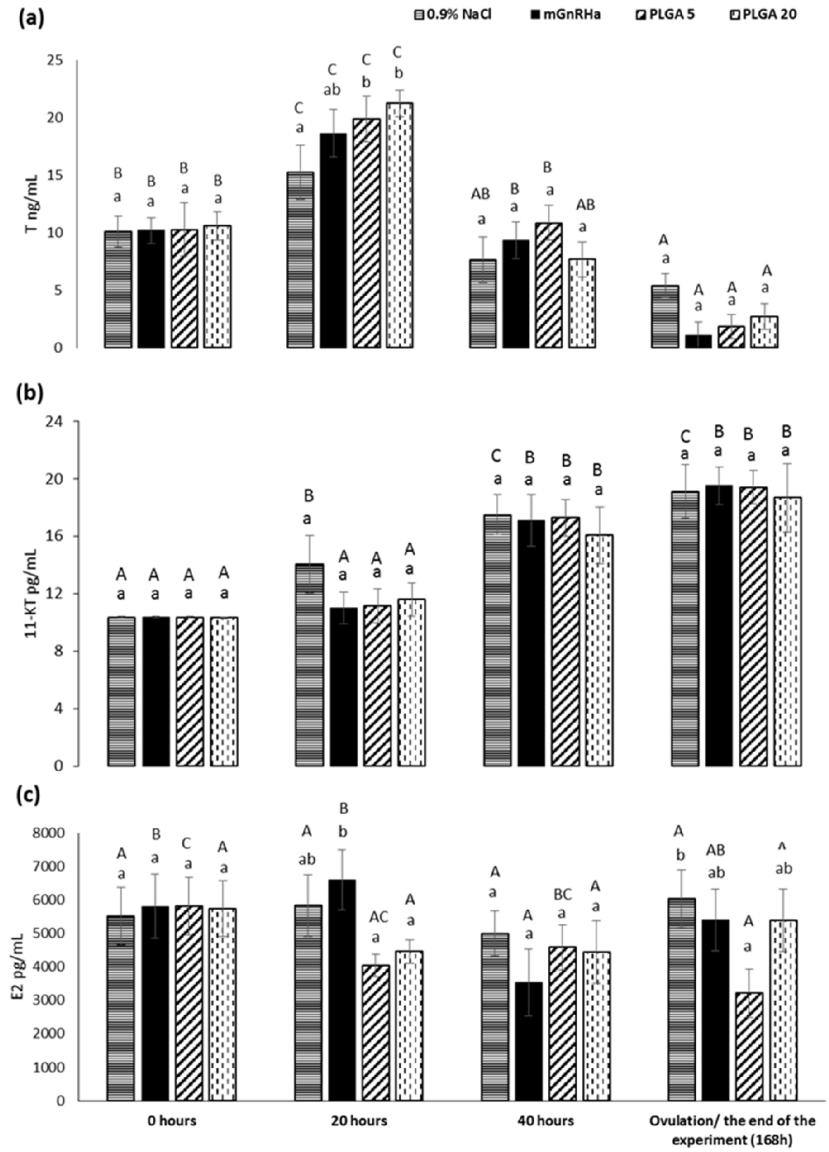

Figure 3. Changes in plasma levels of testosterone (**a**), 11-ketotestosterone (**b**), and 17β-estradiol (**c**) in groups injected with 0.9% NaCl at 1 mL/kg; single injection of mammalian gonadotropin-releasing hormone agonist (mGnRHa) at 20 μg/kg; 5 μg mGnRHa/kg BW embedded in PLGA microparticles (PLGA 5); and 20 μg mGnRHa/kg BW embedded in PLGA microparticles (PLGA 20). [a,b] Significant differences among groups at a sampling point are indicated by lower case letters (one-way ANOVA). [A,B,C] Significant differences within an experimental group are indicated by upper case letters (one-way ANOVA).

4. Discussion

The present study was carried out to develop and investigate the efficacy of microparticle-sustained delivery of mGnRHa. The use of PLGA microparticles at 5 μg/kg BW induced 100% ovulation, significantly higher than in mGnRHa group (40%).

High effectiveness of low mGnRHa dose incorporated in PLGA is an interesting result because higher acute doses of GnRHa (up to 100 µg/kg) are usually used to induced ovulation in pikeperch [27,28]. Encapsulation of GnRHa increases its stability [29], which otherwise has a short lifetime in blood [30]. Sustained release of mGnRHa shows advantages compared to conventional forms, containing controlled release, low toxicity, better efficacy and avoids the necessity of using multiple injections [16]. The high response of pikeperch to sustained mGnRHa release from PLGA microparticles compares well with studies of systems for prolonged GnRHa release in other fish species. A positive effect of sustained mGnRHa release on fish reproduction has been confirmed common carp *Cyprinus carpio* [21], common snook *Centropomus undecimalis* [31], spotted rose snapper *Lutjanus guttatus* [32], greater amberjack *Seriola dumeril* [33] Atlantic cod *Gadus morhua* [34], starry flounder *Platichthys stellatus* [35] and many more [17]. However, sustained GnRHa release systems are commonly used in marine fish species and some salmonids [17] but are understudied in freshwater fish species. We confirm that this route of hormone administration is beneficial to further progress in managing reproduction of freshwater fish species.

Production of eggs of inconsistent quality [36] is a major bottleneck in artificial reproduction of pikeperch [14]. Effectivity to induce ovulation in pikeperch by a single injection of mGnRHa is significantly outperformed by two consecutive mGnRHa injections [37,38]. Nevertheless, a single injection is preferable to reduce the stress connected with hormone treatment and manipulation, which may be associated with poorer egg quality [15]. In some species, higher GnRHa dosages can lead to low ovulation rate [39,40] and additionally cause production of low-quality eggs [41,42]. Our obtained hatching rate of 53.4–68.0% for all groups was not significantly affected by hormone treatment. Similar hatching rate (51–71%) in pikeperch was confirmed in semi-artificial propagation after stimulation with hCG [7] as well as in an experiment focused on out-of-season pikeperch spawning (49–64%) [43]. Our results, showing no difference among groups, indicate that the route of mGnRHa administration affects the ovulatory event but not egg quality. Other researchers also reported no differences in fertilization and hatching rate, and thus conclude that the long-term elevated LH levels induced caused by sustained GnRHa delivery does not adversely affect the quality of eggs [44,45]. A positive influence of sustained GnRHa release on the number of eggs was confirmed in some fish species, such as red porgy *Pagrus pagru* [46], common carp [21], turbot *Scophthalmus maximus* [47], and yellowtail flounder *Pleuronectes ferrugineus* [48]. Nevertheless, no differences were observed in terms of absolute and relative fecundity in our study. The same findings were confirmed in other studies, where no effect of various hormonal treatments and dosages on the pikeperch fecundity was noticed [28,38].

Latency period was not significantly modified by PLGA hormonal treatments compared to mGnRHa group and was in the range of 94.6 $\pm$ 13 h. Similar latency was reported in female pikeperch after a single injection of mGnRHa [28]. Shorter latency is often observed in females treated with gonadotropins acting directly on the gonads (carp pituitary extract, hCG) compared to treatments acting on the brain (GnRHa) [12]. Studies to assess modulation of the latency period in percids via administration of certain spawning agents have shown negative results [8,14,49].

To improve artificial reproduction of pikeperch, it is crucial to understand the physiological changes during the final maturation in cultured fish [13]. Decrease of reproductive capacity in domesticated pikeperch [50] is due to depression of sex steroids during oogenesis. Lower levels of E2 in domesticated pikeperch females may be due to its ineffective conversion from T compared to that in wild females [51,52]. In fish with synchronous ovarian development, the concentration of E2 increases during vitellogenesis while T increases later, and both levels drop during final oocyte maturation and ovulation [53]. This trend was confirmed in our experiment in females stimulated with mGnRHa. In the groups treated with PLGA microparticles, the level of E2 showed a non-significant downward trend with no increasing levels observed. As E2 is known to inhibit the entry of oocytes into the final oocyte maturation in a range of fish species, we assume that lower E2 after

PGLA injection might be linked to the increase of ovulation, at least with the dose of 5 µg GnRHa/kg BW.

In the present study, plasma T levels showed significant increasing values after 20 h in the groups stimulated with PLGA microparticles compared to the control group, indicating stimulation of gonadotropin-mediated sex steroid secretion by the gonad. The hormone treatment significantly stimulated production of T, which decreased toward ovulation. This trend has been reported in other studies of cultured pikeperch [14]. The results of our study show that hormone stimulation, irrespective of administration method, is effective in stimulation of T secretion in pikeperch. As E2 can be converted directly from T [51], our findings suggests that the lower and unstable concentrations of E2 may be caused by insufficient conversion from T.

The level of 11-KT increased after 40 h in all groups regardless of treatment. The role of 11-KT in vitellogenesis and ovulation is scarcely studied, and further investigation is needed. We found higher levels of 11-KT in the end of the experiment in female pikeperch, which may indicate an effect on ovulation [54]. Whether it is T or 11-KT, the absence of and effect of treatment type on androgen regulation suggests that the route of GnRH exposure does not influence the androgen secretion and that the improvement of the ovulation rate with PGLA 5 is not due to differential release.

The PLGA microparticles released GnRHa for up to 5 days at temperature 15 °C. Hormonal treatment employing PLGA microparticles may have important benefits over acute hormone injections or solid implants. In contrast with cholesterol [17] and EVAc implants [44], the PLGA polymer is biodegradable and breaks down into lactic and glycolic acid, which enter the Krebs cycle. Following that, it is further decomposed to water and carbon dioxide [54], which can be an important factor in broodstock that may be further sold on the market. Another advantage over solid implants is that PLGA microparticles dose can be simply adjusted, which allows the treatment of species from 20 kg to 20 g [20]. Moreover, due to the prolonged GnRHa release from the PLGA microparticles, the necessity of using re-injections and repetitive handling can be eliminated [16]. It is important to note that the tank condition may affect fish reproduction. This was not reflected in the methodology of the study and should not be omitted in future studies. Future studies should give special importance in using a control group injected with pure PLGA microparticles as well as the immune response of the fish to treatment. Further investigation of efficacy of PLGA microparticles in inducing ovulation and spermiation, gametes quality, and the steroid feedback will be studied in various fish species.

5. Conclusions

A low dose of mGnRHa in PLGA microparticles is effective in inducing ovulation. The sustained release of GnRHa encapsulated in PLGA microparticles results in acceptable reproductive output, establishing its potential as a tool for the induction of ovulation in pikeperch. The ease of producing the microparticles and potential for controlled GnRHa release may be effective in overcoming reproductive dysfunction in cultured fish.

Author Contributions: Conceptualization, J.K. and P.P.; methodology, J.K.; software, J.K.; validation, J.K., S.M., and M.H.; formal analysis, J.K.; investigation, J.K. and T.P.; resources, J.V. and M.H.; data curation, S.M.; original draft preparation, J.K.; review and editing, T.P.; illustration, J.K.; supervision, T.P.; project administration, P.P.; and funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministry of Agriculture of the Czech Republic, grant number NAZV QK1810221.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and in accordance with national and international guidelines for the protection of animal welfare (EU-harmonized Animal Welfare Act of the Czech Republic). The experimental unit is licensed (No. 44216/2015-MZE-17214, valid from 18 January 2018 to 30 June

2021, within project NAZV QK1810221) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

EFFECTS OF GONADOTROPIN-RELEASING HORMONE AGONIST ADMINISTERED IN MICROPARTICLES ON SPERM QUALITY AND QUANTITY, AND PLASMA SEX STEROID LEVELS IN NORTHERN PIKE

Knowles, J., Boryshpolets, S., Kholodnyy, V., Rahi, D., Vysloužil, J., Muselík, J., Stejskal, V., Kouřil, J., Podhorec, P., 2022. Effects of gonadotropin-releasing hormone agonist administered in microparticles on sperm quality and quantity, and plasma sex steroid levels in northern pike. Animal 16, 100430.

My share on this work was about 50%.

Animal 16 (2022) 100430

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effects of gonadotropin-releasing hormone agonist administered in microparticles on sperm quality and quantity, and plasma sex steroid levels in northern pike

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ARTICLE INFO

Article history:
Received 21 April 2021
Revised 20 November 2021
Accepted 22 November 2021
Available online 24 December 2021

Keywords:
Fish
Maturation
Sperm motility
Sperm production
Sustained drug release

ABSTRACT

Artificial reproduction of northern pike *Esox lucius* is impeded by the likelihood of obtaining only a small volume of sperm of inconsistent quality. A controlled-release hormone delivery system has the potential to enhance sperm production while avoiding multiple injections The objective of this study was to investigate the effects of mammalian gonadotropin-releasing hormone agonist (**mGnRHa**) incorporated into poly(lactic-co-glycolic acid) (**PLGA**) microparticles on milt production, spermatozoon characteristics, and secretion of 17β-estradiol (**E2**), 11-keto testosterone (**11-KT**), and testosterone in northern pike. Fish were divided into four groups and injected with 2 mg/kg BW carp pituitary extract (**CPE**), 20 μg/kg BW mGnRHa in PLGA microparticles, or 20 μg/kg BW mGnRHa plus 20 mg/kg BW metoclopramide (**MET**) in PLGA microparticles (PLGA + MET), along with a control group injected with 1 ml/kg 0.9% NaCl. At 48 h postinjection, the volume of milt produced was significantly greater in groups treated with CPE and PLGA + MET than in other groups. At 96 h postinjection, all hormone-treated groups exhibited significantly higher spermatozoon average velocity than recorded in the control group. Spermatozoon motility was significantly increased ($P < 0.05$) in the CPE and PLGA groups compared to baseline values. All treated groups showed significantly lower levels of 11-KT after the hormone injection compared to baseline values and to controls. Plasma testosterone levels increased in all hormone-treated groups. The use of PLGA microparticles, with or without metoclopramide, is suitable for use as a carrier of hormone treatments to regulate spermiation in mature northern pike.

Implications

Hormone treatment to induce spawning in cultured fish, often through multiple injections, can induce stress affecting the quantity and quality of obtained gametes. A poly(lactic-co-glycolic acid) microparticle sustained-release system to administer gonadotropin-releasing hormone agonist to northern pike positively affected sperm volume, total spermatozoon count and concentration, and spermatozoon velocity. The treatment, with or without dopamine inhibitor, was associated with spermatozoon motility, relative milt weight and plasma sex steroid levels similar to values observed following acute injection of carp pituitary extract. These results establish the potential of poly(lactic-co-glycolic acid) microparticles as a novel tool for hormone treatment in fish.

Introduction

Northern pike *Esox lucius* is a keystone piscivorous fish inhabiting cold freshwater ecosystems of the northern hemisphere. It is a popular game fish (Miller and Kapuscinski, 1997) and has been extensively cultivated in Europe and Asia for decades (Balik et al., 2006). Its high economic value as a food fish and ready acceptance of a formulated pellet diet make it a promising candidate for diversification of intensive aquaculture (Kucska et al., 2005).

The spawning period in northern pike occurs at water temperature of 7–10 °C (Billard and Marcel, 1980). In artificial reproduction, sperm is primarily obtained by killing males to collect testicular milt (Lahnsteiner et al., 1998) or by stripping via abdom-

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https://doi.org/10.1016/j.animal.2021.100430
1751-7311/

inal massage (Billard and Marcel, 1980), which usually yields only a small volume of milt and carries a risk of urine, blood, or faece contamination which ultimately lowers the quality of obtained sperm (Hulak et al., 2008). To evaluate the quality of broodstock, fish milt density and volume along with spermatozoon motility and velocity are commonly analysed parameters (Gage et al., 2004; Hulak et al., 2008; Bondarenko et al., 2018).

The intensive production of northern pike fry generally relies on wild broodstock captured from culture ponds and transported to the hatchery. This manipulation may induce a stress response that can negatively affect spermiation, accelerating or delaying time of reproduction (Schreck, 2010), lead to low volume and poor quality sperm, and, in females, lack of oocyte maturation and ovulation and low quality eggs (Cejko et al., 2016).

Hormone therapy is commonly used to enhance spermiation and sperm production in aquaculture. Dopaminergic inhibition is present in some fish species, and a combination of gonadotropin-releasing hormone agonist (**GnRHa**) and dopamine antagonists (metoclopramide, pimozide or domperidone) can be used to induce ovulation or spermiation in fish (Szabó, 2003). A positive effect on spermiation of a combination of GnRHa and dopamine was observed in pike (Cejko et al., 2018); while GnRHa treatment alone did not induce spermiation in this species (Billard and Marcel, 1980). Administration of GnRHa in multiple injections is often used when gamete maturation requires long-term hormone exposure (Schreck, 2010), but repetitive manipulation of broodstock may cause stress and negatively affect gamete quantity and quality. Use of prolonged-release drug delivery systems is worthwhile option, especially when broodstock are kept outdoors in ponds or sea cages (Santamaria et al., 2013).

Cholesterol pellets (Ibarra-Castro et al., 2017), co-polymers of ethylene and vinyl acetate (Jerez et al., 2018), and chitosan nanoparticles (Rather et al., 2013) have been used successfully as drug carriers in fish. A recently developed technique incorporates GnRHa into microparticles of poly(lactic-co-glycolic acid) (**PLGA**), a biodegradable polymer currently in use as a carrier of pharmaceutical products (Matejkova and Podhorec, 2019). A range of encapsulated agents in PLGA have been successfully used in fish, including bacterial (Altun et al., 2010), viral (Adomako et al., 2012), and parasitic antigens (Harikrishnan et al., 2012). With respect to fish reproduction, PLGA microparticles with incorporated mammalian gonadotropin-releasing hormone agonist (**mGnRHa**) were successfully used for induction of ovulation of peled *Corgeonus peled* (Knowles et al., 2021). To the best of our knowledge, this was the first published report of studies employing PLGA microparticles in fish reproduction.

The objective of the present study was to determine the efficacy of PLGA microparticles as carriers for sustained release of GnRHa, with or without separate injection of a dopamine inhibitor, compared to acute hormone injection of carp pituitary extract (**CPE**) and to assess its effects on quantity and quality of spermatozoa in northern pike.

Material and methods

In mid-March 2019, northern pike males ($n = 40$) (1 430 ± 301 g) were collected from earth ponds at the Faculty of Fisheries and Protection of Waters (South Czechia) and transported to the experimental facility of the Faculty of Fisheries and Protection of Waters (Vodnany) (49°N, 14°E). Fish were held in 1 000 l tanks (pH, 7.2–7.8; oxygen saturation, 90%) freshwater at 11 ± 1 °C for 7 days of acclimatisation. After acclimatisation, fish were randomly divided into four groups of ten, three receiving spermiation hormone stimulation plus a control group (Table 1).

Table 1
Selected preparations for hormonal stimulation of male northern pike.

Group	Treatment	Dose
Control	0.9% NaCl	1 ml/kg
CPE	Carp pituitary extract	2 mg/kg
PLGA	PLGA + GnRHa	20 μg GnRHa/kg
PLGA + MET	PLGA + GnRHa + metoclopramide	20 μg GnRHa + 20 mg MET/kg

Abbreviations: CPE = carp pituitary extract; PLGA = microparticles of poly(lactic-co-glycolic acid); MET = Metoclopramide; GnRHa = gonadotropin-releasing hormone agonist.

Fish received a single intraperitoneal injection of: 0.9% NaCl (Braun Melsungen AG, Germany) (C; 1 ml/kg), carp pituitary extract (FFPW Vodnany, Czech Republic) (CPE; 2 mg/kg), GnRHa-embedded PLGA microparticles (PLGA; 20 μg GnRHa/kg), or combination of GnRHa-embedded PLGA microparticles plus a separate injection of dopamine-inhibitor metoclopramide (**MET**) (Sigma-Aldrich, USA) (PLGA + MET; 20 μg GnRHa + 20 mg MET/kg). The preparations were homogenised in 0.9% NaCl, and each fish received 1 ml/kg BW using a 2 ml syringe fitted with a 0.9 × 40 m m needle. The doses of CPE and GnRHa were based on the findings of several studies (Billard and Marcel, 1980; Cejko et al., 2018; Cejko et al., 2020) and the metoclopramide dose on that applied in studies of other freshwater fish species (Cejko and Kucharczyk, 2015; Podhorec et al., 2017).

After the hormone stimulation, each group was placed in separate 700 l tanks and held under a constant water temperature of 12 ± 1 °C, until the end of the experiment.

Poly(lactic-co-glycolic acid) microparticle preparation

Microparticle preparation used a standard water-in-oil-in-water combination. Ten mg of alarelin acetate (APExBIO, Houston, TX, USA) was dissolved in 1.5 g of warmed (50 °C) 9.1% gelatine solution. PLGA RESOMER® RG 753H (800 mg) (Evonik, Darmstadt, Germany) was dissolved in 5 ml dichloromethane (Penta, Prague, Czech Republic) (oil phase). Both water and oil phases were premixed by vortexing for 30 s and then homogenised for 60 s (Ultra-Turrax T25, Ika Werke, Staufen Im Bresgau, Germany). The resulting water-in-oil emulsion was pre-mixed for 60 s on a homogeniser with 12 g of warmed (50 °C) 1% poly(vinyl alcohol) solution (Mw 31 000–50 000, 98–99% hydrolysed; Sigma–Aldrich, St. Louis, MO, USA) to create a concentrated double emulsion w1/o/w2. The concentrated emulsion was immediately poured into 200 g of 0.1% poly(vinyl alcohol)/2.0% NaCl. The resulting emulsion was stirred for 2 hours to completely evaporate dichloromethane. Particles were collected by centrifugation, re-suspended in purified water, and lyophilised.

Sperm collection

Sperm was collected at 0, 48, and 96 h posthormone stimulation. Fish were anaesthetised with clove oil at 0.05 ml/l water, and sperm was stripped using gentle abdominal massage directly into pre-weighed 5 ml syringes, being careful to avoid contamination by mucus, urine, faeces, or blood. Sperm samples were kept at 0–2 °C during transportation to the laboratory and throughout the motility analysis assays.

Determination of sperm quality and quantity

Spermatozoon motility was assessed by mixing sperm ~1:1 000 in activation medium containing 10 mM Tris buffer (pH 8.4) supplemented with 0.25% pluronic F-127 (Sigma-Aldrich) under a

microscope. To obtain a cell concentration of 50–150 cells in the observation field, sperm was added to a microscopic slide using a tip of an injection needle and mixed with 40 μl activation medium. The temperature of the slide was maintained at 11 ± 1 °C on Thermostage (CEMIK, Poland).

Video recordings at 25 frames/s for 60 s were made using a light microscope with digital camera (IDS Imaging Development System GmbH, Germany), a negative phase-contrast condenser and 10× lens. Each sample was recorded in triplicate (technical replicate). Video records were saved in Audio Video Interleave format and further analysed. Spermatozoon average-path velocity (**VAP**), curvilinear velocity (**VCL**), and straight-line velocity (**VSL**) at 10 s postactivation were obtained, as well as percent of motile cells (motility rate, %). These parameters are commonly used as quality indices in fish sperm studies. The analysis was made with ImageJ software (National Institutes of Health, USA) and CASA plugin (Wilson-Leedy and Ingermann, 2007; Purchase and Earle, 2012).

Spermatozoon concentration was assessed using a Bürker chamber. Sperm was diluted 1:10 000 in physiological solution in two steps, placed in the chamber, and the calculation was conducted within 5 min. Each sample was counted in triplicate. Spermatozoon concentration was expressed in billions/ml.

A sperm sample from each male was weighed (Kern, PCD 300-3), and its weight (g) was used as a proxy for volume (ml), allowing calculation of the total number of spermatozoa per fish (spermatozoon concentration × sperm weight × 10^9 spermatozoa/ml) and relative milt weight (g milt/kg fish BW).

Blood sampling and steroid assay

A 2 ml blood sample was collected into a 5 ml heparinised syringe by caudal venipuncture at 0, 48, and 96 h postinjection and centrifuged at 1 500*g* for 10 min at 4 °C. Plasma was stored at −20 °C until analysis. Sex steroids in plasma were analysed by ELISA method twice in each sample.

Testosterone (ng/ml) was assayed in 25 μl of plasma using the DIAsource Testosterone ELISA kit (DIAsource, KAPD1559) according to the manufacturer's instructions. Samples were tested for dilution accuracy and when necessary, diluted 1:2 using Calibrator 0 as diluent. Sensitivity of the assay was 0.083 ng/ml. Intra-assay CV was 18.9%.

Androgen 11-keto testosterone (**11-KT**, ng/ml) was assayed in 50 μl of plasma diluted 1:10 in ELISA buffer using the Cayman 11-KT EIA kit (Cayman, 582751). Sensitivity was 1.3 pg/ml, CV intra-assays were 2.6, 1.9, and 8.4%, and CV inter-assays were 9.4, 9.6, and 12.1% for 0.78, 6.25, and 100 pg/ml, respectively.

17β-estradiol (**E2**, ng/ml) was assayed in 50 μl of plasma using the DIAsource E2-ELISA kit (DIAsource, KAP0621). When required, samples were diluted 1:20–1:70. Sensitivity was 5 pg/ml, CV intra-assays were 3, 2.3, and 2.6%, and CV inter-assays were 4.7, 4.1, and 2.4% for 37, 118, and 880 pg/ml, respectively.

Data analysis

All data were analysed by Statistica 12 Cz (StatSoft, Tulsa, USA). Normality (Shapiro-Wilk test) and homogeneity (Bartlett test) of data were tested. Significant differences among groups and significant differences for each group throughout the experimental period were analysed by one-way ANOVA followed by Tukey HSD test. Results were considered significant at $P < 0.05$. Data collected at 0 h were used for assessment of the baseline values for each parameter and group separately. All data are presented as mean ± SEM.

Results

Sperm production and spermatozoon concentration

The quantity of milt was significantly higher 48 h posthormone treatment in the CPE ($P = 0.0001$) and PLGA group ($P = 0.0263$) compared to baseline values. There were no significant differences among tested groups in relative milt weight at any collection time ($P > 0.05$, Fig. 1a).

At 48 and 96 hr, a significantly higher total number of spermatozoa was obtained in the PLGA + MET group compared to other treatments and baseline values ($P = 0.0427$, Fig. 1b). A significantly greater number of spermatozoa compared to baseline values were also observed with CPE treatment ($P = 0.0207$).

Spermatozoon motility

The VAP 10 s postactivation of spermatozoa collected at 0 h did not differ significantly among groups ($P > 0.05$) and showed a mean value of 110.14 ± 2.31 μm/s. The hormone-treated groups exhibited significantly higher VAP compared to the control group 96 h postinjection ($P = 0.0028$, Fig. 2a).

The VCL of spermatozoa collected in the beginning of the experiment did not differ significantly among groups ($P > 0.05$), and mean of all groups was 120.87 ± 1.96 μm/s 10 s postactivation. The PLGA group exhibited significantly higher VCL 48 ($P = 0.0048$) and 96 h postinjection ($P = 0.0282$) compared to the baseline values. The controls showed significantly lower VCL at 96 h compared to other sampling points ($P = 0.0016$), as well as to other groups at the same sampling time ($P = 0.0013$, Fig. 2b).

The VSL 10 s postactivation of spermatozoa collected at 0 h did not differ significantly among tested groups (group mean 87.14 ± 2.03 μm/s; $P > 0.05$). Spermatozoon of the PLGA group exhibited significantly higher VSL at 96 h postinjection compared to other sampling points ($P = 0.0203$), as well as to that of other groups collected at 96 h ($P = 0.0078$, Fig. 2c).

The average motility rate of spermatozoa at 0 h was significantly lower in CPE compared to other treatments ($P = 0.0046$), with the rate increasing significantly 48 h postinjection compared to baseline values ($P = 0.001$). The motility rate in the PLGA group was significantly higher 48 h postinjection compared to the baseline values ($P = 0.004$, Fig. 3).

Serum hormone levels

No significant differences among the treated groups were found in E2 plasma concentration ($P > 0.05$, Fig. 4a). In contrast, the plasma level of testosterone increased in all hormone-treated groups compared to baseline values and to the control group ($P = 0.05$). With no significant differences observed among the hormone-stimulated groups ($P > 0.05$, Fig. 4b), all hormone-treated groups showed significantly lower 11-KT postinjection compared to baseline values ($P = 0.007$) and to the control group ($P = 0.0023$), but no significant differences were found among these groups ($P > 0.05$, Fig. 4c).

Discussion

The present study demonstrates the effectiveness of PLGA microparticles as a GnRHa sustained-delivery system with or without dopamine inhibitor in enhancing quality and quantity of northern pike sperm. The study provides the first comprehensive information with respect to the impact of a PLGA drug delivery system on the levels of primary sex steroids in the blood.

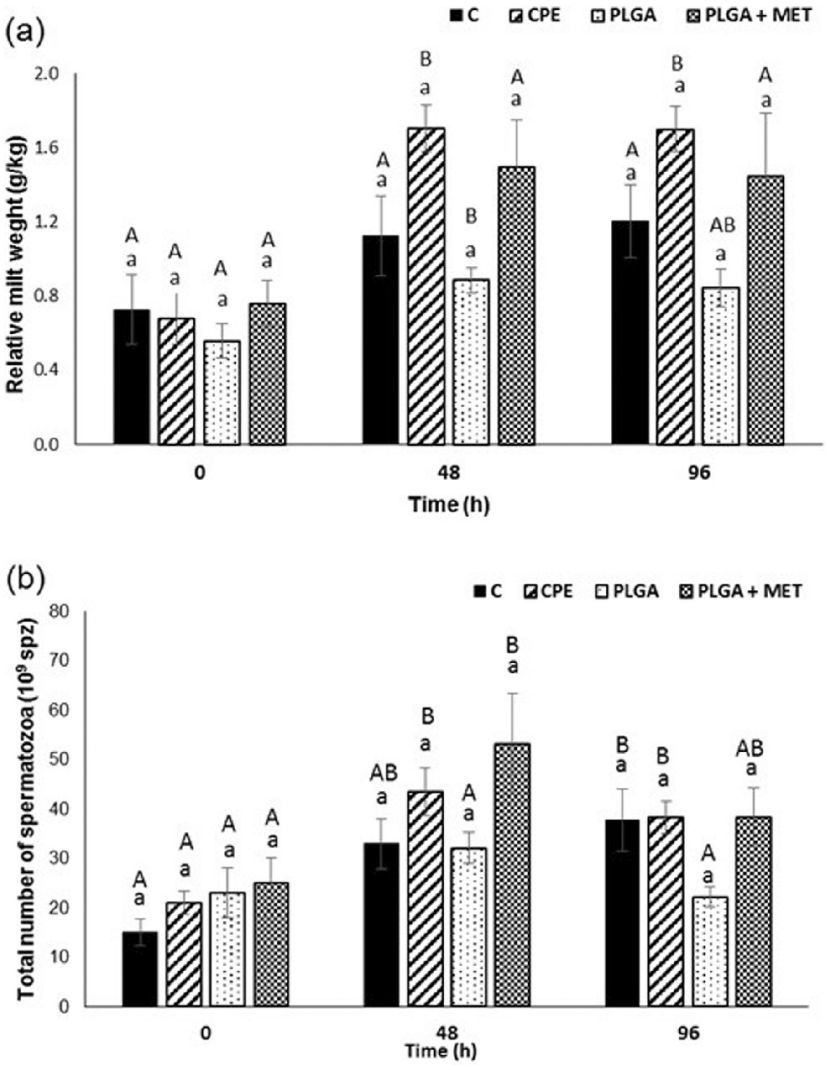

Fig. 1. (a) Relative milt weight and (b) total number of spermatozoa in northern pike relative to time postinjection. Fish were injected with 0.9% NaCl (C) (1 ml/kg, n = 10), carp pituitary extract (CPE) (2 mg/kg, n = 10), GnRHa-embedded PLGA microparticles (PLGA) (20 μg GnRHa/kg, n = 10) and combination of GnRHa-embedded PLGA microparticles plus a separate injection of dopamine-inhibitor metoclopramide (PLGA + MET) (20 μg GnRHa + 20 mg MET/kg, n = 10). Significant differences between groups at a sampling point are indicated by lower case letters (one-way ANOVA, $P < 0.05$). Significant differences within an experimental group over the experimental period are indicated by capital letters (one-way ANOVA, $P < 0.05$). Abbreviations: GnRHa = gonadotropin-releasing hormone agonist; PLGA = microparticles of poly(lactic-co-glycolic acid).

Hormone treatment long has been employed to increase volume and quality of sperm in cultured fish (Mylonas and Zohar, 2001), and GnRHa for stimulation of maturation, ovulation, and spermiation (Zohar, 1989). In many fish species, re-injection of the hormone is essential to promote complete maturation and reproduction (Dabrowski et al., 1994; Slater et al., 1995). Advanced drug delivery systems have been developed to minimise manipulation and attendant stress and to maintain an effective level of the preparation (Mylonas and Zohar, 2001). A recent example is the incorporation of GnRHa into microparticles of biodegradable polymers such as poly(lactic-co-glycolic acid) (Matejkova and Podhorec, 2019).

The weight of milt obtained was significantly higher 48 h postinjection in the CPE and PLGA groups than the baseline values. Similar results were observed in the related species, muskellunge *Esox musquinongy*, in which a higher yield of sperm was reported 72 h post-stimulation with CPE at 3.3 mg/kg BW (Lin et al., 1996). Northern pike produces a lower volume of sperm at the beginning and the end of the reproductive season with significantly higher levels midway in the spawning period (Bondarenko et al., 2018). Higher milt production was reported in northern pike after a single injection of partially purified salmon gonadotropin as well as with crude CPE and fresh pike pituitary extract, whereas GnRHa alone did not increase milt production (Billard and Marcel, 1980). Administration of GnRHa in an implant significantly increased milt production in Atlantic cod *Gadus morhua* compared to controls irrespective of dose (Garber et al., 2009).

We found the total numbers of spermatozoa to be significantly increased in PLGA + MET and CPE groups compared to the baseline values. The highest spermatozoon count was observed in

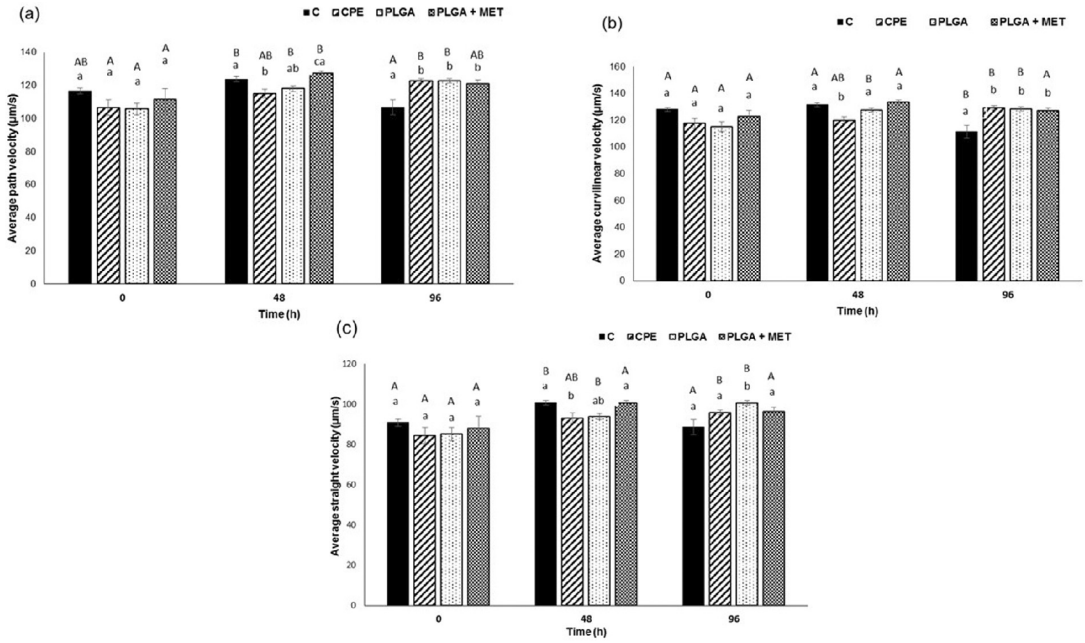

Fig. 2. (a) Average path velocity, (b) average curvilinear velocity, and (c) average straight-line velocity at 10 s postactivation in northern pike relative to time postinjection. Fish were injected with 0.9% NaCl (C) (1 ml/kg, n = 10), carp pituitary extract (CPE) (2 mg/kg, n = 10), GnRHa-embedded PLGA microparticles (PLGA) (20 μg GnRHa/kg, n = 10) and combination of GnRHa-embedded PLGA microparticles plus a separate injection of dopamine-inhibitor metoclopramide (PLGA + MET) (20 μg GnRHa + 20 mg MET/kg, n = 10). Data are presented as mean ± SEM. Significant differences between groups at a sampling point are indicated by lower case letters (one-way ANOVA, $P < 0.05$). Significant differences within an experimental group throughout the experimental period are indicated by capital letters (one-way ANOVA, $P < 0.05$). Abbreviations: GnRHa = gonadotropin-releasing hormone agonist; PLGA = microparticles of poly(lactic-co-glycolic acid).

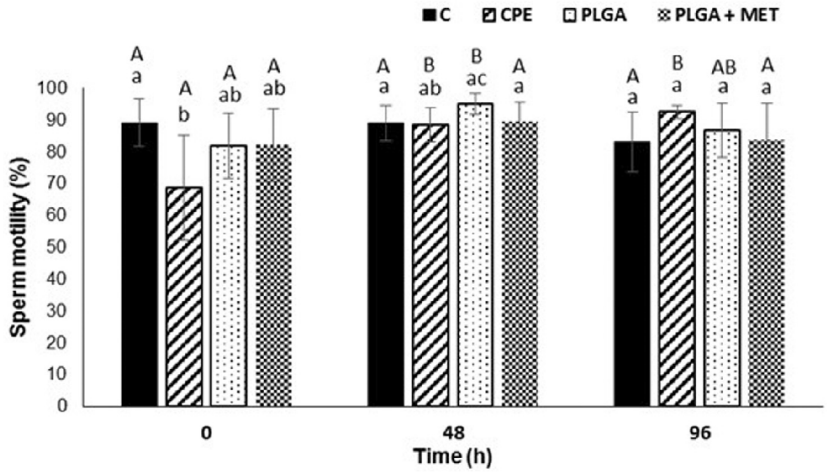

Fig. 3. Effect of hormonal treatments on percent of motile spermatozoa of northern pike in stripped samples at time posthormone injection. Fish were injected with 0.9% NaCl (C) (1 ml/kg, n = 10), carp pituitary extract (CPE) (2 mg/kg, n = 10), GnRHa-embedded PLGA microparticles (PLGA) (20 μg GnRHa/kg, n = 10) and combination of GnRHa-embedded PLGA microparticles plus a separate injection of dopamine-inhibitor metoclopramide (PLGA + MET) (20 μg GnRHa + 20 mg MET/kg, n = 10). Data are presented as mean ± SEM. Significant differences among groups at a sampling point are indicated by lower case letters (one-way ANOVA, $P < 0.05$). Significant differences within an experimental group throughout the experimental period are indicated by upper case letters (one-way ANOVA, $P < 0.05$). Abbreviations: GnRHa = gonadotropin-releasing hormone agonist; PLGA = microparticles of poly(lactic-co-glycolic acid).

PLGA + MET (53.18 ± 10.21 10^9/ml). These results are more pronounced than those obtained in muskellunge, in which neither human chorionic gonadotropin nor CPE resulted in significant differences in sperm count 72 h postinjection. The number was significantly lower in the CPE group compared to human chorionic gonadotropin and controls at 96 h (Lin et al., 1996). The concentration of stripped sperm in northern pike can be impacted by dilution with urine (Hulak et al., 2008), which may be difficult to avoid given the proximity of the sperm duct to the ureter (Dietrich et al., 2005).

We found a mean spermatozoon motility percentage of 86 ± 1.01% for all groups over all sampling times. This value is

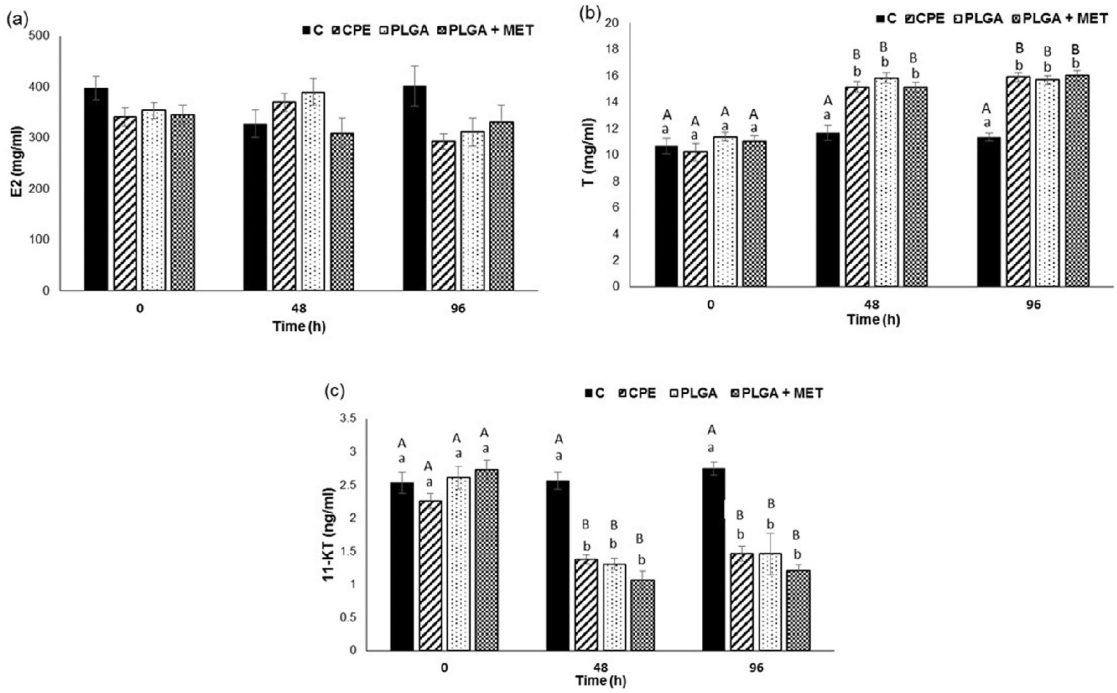

Fig. 4. Effect of hormonal treatments on plasmatic concentration of (a)17β-estradiol (E2), (b) testosterone (T) and (c)11-keto testosterone (11-KT) in northern pike. Fish were injected with 0.9% NaCl (C) (1 ml/kg, n = 10), carp pituitary extract (CPE) (2 mg/kg, n = 10), GnRHa–embedded PLGA microparticles (PLGA) (20 μg GnRHa/kg, n = 10) and combination of GnRHa-embedded PLGA microparticles plus a separate injection of dopamine-inhibitor metoclopramide (PLGA + MET) (20 μg GnRHa + 20 mg MET/kg, n = 10). Data are presented as mean ± SEM. Significant differences between groups at a sampling point are indicated by lower case letters (one-way ANOVA, $P < 0.05$). Significant differences within an experimental group throughout the experimental period are indicated by upper case letters (one-way ANOVA, $P < 0.05$). Abbreviations: GnRHa = gonadotropin-releasing hormone agonist; PLGA = microparticles of poly(lactic-co-glycolic acid).

higher than the highest observed motility (45–50%) over the entire northern pike reproductive season (Bondarenko et al., 2018). In a study of muskellunge, hormone stimulation did not change the spermatozoon motility rate, and 90–100% motility was reached during the experiment (Lin et al., 1996). GnRHa administered as sustained-release implants did not affect motility rate in white bass *Morone chrysops* (Mylonas et al., 1997) or winter flounder *Pleuronectes americanus* (Shangguan and Crim, 1999) but was associated with increased motility in yellowtail flounder *Pleuronectes ferrugineus* (Clearwater and Crim, 1998), Atlantic halibut *Hippoglossus hippoglossus* (Vermeirssen et al., 2004), and Atlantic Cod (Garber et al., 2009).

In our experiment, VAP was significantly higher 96 h post-stimulation in all hormone-treated groups compared to controls, with no significant differences among treatments. The mean VAP of all hormone-treated groups at the conclusion of the experiment was 121.16 ± 0.9 μm/s. Slightly higher VAP compared to our experiment (132.5 ± 19.1 μm/s) was observed in spermatozoa of wild northern pike (Cejko et al., 2016). Previous studies in fish have shown that higher spermatozoon velocity to be positively correlated with fertilisation rate (Gage et al., 2004).

The VCL at 96 hours postinjection was significantly greater in all hormone-stimulated groups compared to controls. The VSL in CPE and PLGA groups 96 h post-stimulation was significantly increased from the baseline values. Curvilinear velocity and straight-line velocity were not affected by stimulation by CPE (2 mg/kg), Ovopel (0.5 pellet/kg), or Ovaprim (0.25 ml/kg) in crucian carp *Carassius carassius* (Cejko et al., 2013). The values of VCL and VSL in spermatozoa of wild mature northern pike were reported to be 159.4 ± 27.9 μm/s and 59.9 ± 12.3 μm/s, respectively (Cejko et al., 2016). The fact that wild northern pike exhibited better velocity than cultured fish may show the potential for increasing these values in domesticated northern pike by hormonal treatments.

Plasma testosterone levels increased in all hormone-treated groups after stimulation. This trend was also observed with CPE, Ovopel, and GnRH in sterlet *Acipenser ruthenus* (Alavi et al., 2012). Similar results were obtained in European seabass *Dicentrarchus labrax* with several GnRHa sustained-delivery systems increasing testosterone compared to controls (Mañanós et al., 2002). These results suggest that, in addition to their androgenic role during spermatogenesis, testosterone levels are associated with spermiation. During this period, the release of testosterone into the bloodstream may be related to feedback actions in the brain and pituitary, as well as associated with its action as a precursor of the synthesis of other steroid hormones directly involved in the control of spermiation (Nagahama, 2002).

High inter-species variability in the concentration of 11-KT is commonly observed in male teleosts (Barcellos et al., 2002; Golshan and Alavi, 2019). In some fish species, the concentration of 11-KT exhibits increase towards maturation, with the highest concentration seen at the onset of the reproduction (Agulleiro et al., 2007; Schiavone et al., 2012), suggesting 11-KT as a key player at the final maturation process inducing spermiation (Barcellos et al., 2002). However, some fish species also exhibit a decreasing trend of 11-KT during spermiation (Scott et al., 1984; Mañanós et al., 2002). Levels of 11-KT and testosterone can be negatively influenced by stress via its impact on gonadotropin production (Castranova et al., 2005). In our study, 11-KT significantly decreased in all treated groups irrespective of hormone preparation. We believe that a negative impact of stress on 11-KT concen-

tration can be excluded, since testosterone levels increased during the study. These findings support the lack of a role for 11-KT in maintaining spermiation, similar to reports in other fish species (Borg, 1994; Miura et al., 1994).

No significant differences among the treated groups were found in E2 plasma concentration during the experiment. Little is known about the role of E2 in regulation of fish spermatogenesis and spermiation (Zohar and Mylonas, 2001), although it is thought to regulate spermatogonial stem cell renewal (Miura et al., 1994). In male fish, E2 is generally present in blood serum in low concentrations with increase seen in the first months of the reproductive cycle, followed by decrease towards spermiation (Fitzpatrick et al., 1986; Amer et al., 2001).

The PLGA microparticle stimulation shows a positive effect on spermatozoon parameters including count and velocity. The efficacy of PLGA microparticle treatment with respect to relative milt weight, spermatozoon motility, and sex steroid plasma level is similar to that obtained with acute CPE injection. It avoids the disadvantages of CPE, including the potential for transmission of disease from donor fish to the recipient. High species specificity of luteinising hormone is a concern (Zohar and Mylonas, 2001). The PLGA microparticle treatment allows a controlled and homogenous physiological response (Vysloužil et al., 2013) and is approved by the US Food and Drug Administration and European Medicine Agency in diverse long acting systems (Matejkova and Podhorec, 2019). The necessity of multiple injections is eliminated by sustained GnRHa release, which can reduce the stress and contribute to improve broodstock welfare (Mylonas and Zohar, 2001).

Ethics approval

Treatment of animals was carried out according to the authorisation for the breeding and delivery of experimental animals (Reference number: 44218/2015-MZE-1721417OZ14202/2015-17214, valid from 17 August 2015 for 5 years) and the authorisation for the use of experimental animals (Reference number: 2293/2015-MZE-1721416OZ22302/2014-17214, valid from 22 January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia, by the Ministry of Agriculture of the Czech Republic.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

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Declaration of interest

The authors have no conflicts of interest to declare.

Acknowledgements

None.

Financial support statement

This research was funded by the Ministry of Agriculture of the Czech Republic – NAZV project No. QK1810221 and GAJU 099/2019/Z.

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CHAPTER 6

INDUCTION OF SPERMIATION IN STERLET *ACIPENSER RUTHENUS* BY PLGA MICROPARTICLE DELIVERY WITH SUSTAINED ALARELIN RELEASE

Podhorec, P., Knowles, J., Vysloužil, J., Boryshpolets, S., Kubová, K., Rodina, M., Kholodnyy, V., Sotnikov, A., Gela, D., Dzyuba, B., 2021. Induction of spermiation in sterlet *Acipenser ruthenus* by PLGA microparticle delivery with sustained alarelin release. Animals 11, 3305.

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Induction of Spermiation in Sterlet *Acipenser ruthenus* by PLGA Microparticle Delivery with Sustained Alarelin Release

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Simple Summary: Cultured sterlet *Acipenser ruthenus* males do not usually undergo spontaneous spermiation, and if any sperm is obtained without hormone treatment, it is generally of diminished quality. We compared efficacy of stimulation of spermiation with carp pituitary extract to that of 35 μg kg^{-1} or 200 μg kg^{-1} body weight gonadotropin-releasing hormone analogue in a sustained release system. Hormone treatments caused a significant increase in testosterone and 11-ketotestosterone, as well as induced spermiation. The delivery system based on poly (lactic-co-glycolic acid) microparticles with slow release of Alarelin at 35 μg kg^{-1} BW effectively induced spermiation, and was associated with extended sperm collection compared to carp pituitary treatment. The sustained delivery system offers an excellent option for spermiation induction in cultured sterlet, and possibly other sturgeon.

Abstract: Carp pituitary treatment versus poly (lactiac-co-glycolic acid) microparticles with slow release of Alarelin at 35 μg kg^{-1} or 200 μg kg^{-1} body weight to induce spermiation was compared in sterlet *Acipenser ruthenus*. All hormone treatments initially increased testosterone and 11-ketotestosterone, with a subsequent decline in testosterone but consistent high levels of 11-ketotestosterone at 48 and 72 h post-treatment. Spermiation did not differ between hormone-treated groups, and was not detected in controls receiving saline solution. Administration of the carp pituitary led to maximum sperm production 24 h post-treatment, followed by a decrease at 48 h post-treatment, with no sperm obtained at 72 h. The effect of Alarelin at 35 μg kg^{-1} bw and carp pituitary did not differ at 24 and 48 h post-treatment, whereas 200 μg kg^{-1} bw Alarelin was associated with significantly lower spermatozoon concentration 24 h post-treatment compared to carp pituitary, with no difference in milt volume. Higher relative sperm production was observed 48 h after injection of Alarelin at 200 μg kg^{-1} bw compared to carp pituitary. Spermatozoon motility was significantly higher in fish receiving Alarelin at 35 μg kg^{-1} bw than 200 μg kg^{-1} bw. The treatment with optimal effect on inducing spermiation was poly (lactic-co-glycolic acid) microparticles with slow release of Alarelin at 35 μg kg^{-1} bw.

Keywords: sperm; reproduction; sturgeon; sustained release

Citation: Podhorec, P.; Knowles, J.; Vysloužil, J.; Boryshpolets, S.; Kubová, K.; Rodina, M.; Kholodnyy, V.; Sotnikov, A.; Gela, D.; Dzyuba, B. Induction of Spermiation in Sterlet *Acipenser ruthenus* by PLGA Microparticle Delivery with Sustained Alarelin Release. *Animals* **2021**, *11*, 3305. https://doi.org/10.3390/ani11113305

Received: 1 November 2021
Accepted: 17 November 2021
Published: 19 November 2021

1. Introduction

The order Acipenseriformes comprises 27 species, with natural distribution in Eurasia and North America [1]. The population of all sturgeon species has declined drastically due to over-fishing, pollution, and river modifications [2], with all sturgeon species listed

under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1998.

Successful completion of the life cycle of sturgeon in captivity and optimization of production techniques is essential, considering the high demand for viable fingerlings and caviar. The conditions on fish farms differ dramatically from those that broodfish are exposed to in natural habitats. Artificial environments lacking natural spawning stimuli do not induce appropriate endogenous responses from the fish [3], and cultured sturgeon often manifest reproductive dysfunction at the final level of gametogenesis [4].

Traditionally, the dysfunction is overcome by the injection of gonadotropins [carp pituitary suspension (CP), chorionic gonadotropins], to supplement production of endogenous luteinizing hormone (LH) [5]. The discovery of gonadotropin-releasing hormone (GnRH) [6] and its effects introduced a new tool for regulating reproduction in cultured fish [7]. Subsequent production of GnRH analogues (GnRHa) with modified amino acid positions has resulted in multiple increase of GnRHa effectiveness in stimulating LH secretion and ovulation [8].

Most research into male sturgeon reproduction has compared the potency of hormonally active substances dissolved in saline solution in inducing spermiation administered by intramuscular or intraperitoneal injection [9,10]. A single study of efficacy of a sustained release system in inducing spermiation in sturgeon showed negative results [4]. Sustained release of GnRHa is based on prolonged stimulation of pituitary gonadotrophs, leading to elevated secretion of LH into the bloodstream with appropriately stimulated steroidogenesis and improved spermiation [11]. Several sustained release systems are used in aquaculture: ethylene-vinyl acetate copolymer (EVAc) implants [12], solid implantable cholesterol pellets [13], oil emulsion [14], and poly (lactic-co-glycolic acid) microparticles (PLGA) [15]. Long-term sustained release systems have significantly improved sperm production in several marine fish species [16,17]. Their biocompatibility, biodegradability, and potential for administering a precise dose of liquid suspension make PLGA microparticles ideal for encapsulation of peptides like GnRHa [18].

We chose the sterlet Acipenser ruthenus as a model species for this study since, in addition to its small size compared to much bigger anadromous sturgeon and freshwater life history, males reach sexual maturity at three years [9]. Without treatment, sterlet males in captivity, as with most sturgeons, will produce no sperm, or will produce sperm of a lower quality [19].

The goal of the study was to determine the efficacy of a PLGA microparticle delivery system with sustained Alarelin release to induce spermiation in sterlet. Comparison with the most common treatment, carp pituitary suspension, was based on qualitative and quantitative parameters of obtained sperm, supported by stimulated levels of the main androgens.

2. Materials and Methods

2.1. Fish Rearing and Pre-Spawning Water Conditions

Individually tagged five to six-year-old male sterlet were held in aquaculture ponds till January. In January, 40 males of similar body weight 1.58 ± 0.24 kg (mean ± SD, one-way ANOVA, $p = 0.59$) were transferred to an indoor recirculation aquaculture system (water temperature 2 °C), and randomly divided into four groups. Each group ($n = 10$) was placed in a separate 0.8 m^3 tank with a heater and aeration, ensuring optimal dissolved oxygen concentrations (higher than 95% of saturation).

Temperature was set to 5 °C for ten days, and then increased to 14 °C within six days (~+2 °C per day), with a further increase in temperature over the next six days to 15 °C. After 24 h, the experimental treatments were administered.

2.2. Treatment

2.2.1. Synthesis of PLGA Microparticles

The microparticles were prepared by solvent evaporation from a multiple emulsion with Alarelin (APExBIO, Houston, TX, USA). Alarelin is a synthetic polypeptide which acts as gonadotropin-releasing hormone analogue agonist and is highly soluble in water. Resomer 753, a copolymer of lactic acid and glycolic acid (75% polylactic acid and 25% polyglycolic acid), was used as carrier.

Precisely 800 mg of PLGA of the Resomer (Evonik, Darmstadt, Germany) was weighed in a wide-necked tube, and 5 g dichloromethane (Penta, Prague, Czech Republic) was added. The contents were capped and allowed to dissolve. Meanwhile, a 10% gelatin solution (Sigma Aldrich, St. Louis, MO, USA) and 12 g of a 1% polyvinyl alcohol solution (PVA; Sigma Aldrich, USA) was heated in a water bath. Further, 10 mg of Alarelin was weighed into a microcentrifuge tube, 1.5 mL gelatin was added, and vortexed to dissolve the drug. The resulting solution was poured into the wide-necked tube containing PLGA dissolved in dichloromethane, and vortexed again to ensure emulsification. The contents of the tube were homogenized to produce a fine emulsion. Subsequent homogenization with 12 g of 1% PVA solution (T25 basic, IKA-Werke, Staufen, Germany) produced a concentrated water/oil/water emulsion, which was then diluted in 200 mL of 0.1% PVA solution containing 2% NaCl and placed under a shaft stirrer set at 450 rpm. The contents of the wide-mouth tube were poured into the external aqueous phase, and the dichloromethane was evaporated for 2 h. The resulting micro-suspension was filtered through a 250 µm screen for the separation of possible agglomerates. Isolation of the microparticles was then performed by centrifugation at 6000× *g* for 2 min. Excess water was decanted, and the microparticles were collected, stored in a freezer, and subsequently dried by lyophilization.

The content of Alarelin in PLGA microparticles was determined by high-performance liquid chromatography (HPLC). First, the microparticles were dissolved in acetone, and the resulting solution was mixed 1:1 (*v*/*v*) with a phosphate buffer of pH 7.0. The resulting mixture was filtered through a 0.45 µm membrane filter. The mixture was quantified by HPLC (Agilent 1100; Agilent Santa Clara, CA, USA) using a NUCLEODUR 100-5 CN-RP column (150 mm × 4.6 mm, 5 µm). Acetonitrile: 20 mM H_3PO_4 (16:84, *v*/*v*) was used as a mobile phase binary mixture, with an 0.8 mL min^{-1} flow rate at 30 °C, 20 µL of injection sample volume, and a detection wavelength of 220 nm. In the dissolution study, 50 mg of microparticles were suspended in 0.4 mL 1% agarose solution in a glass vial, and cooled to solidify the agarose, after which 800 µL of agarose was added and left to solidify, and 5 mL of phosphate buffer was added. At 4, 24, 48, 72, 96, and 168 h, 2 mL of buffer was collected and filtered through a 0.22 µm membrane filter. The remaining buffer was removed, the vials were washed with 0.5 mL of buffer to remove residue, and 5 mL of fresh buffer was added. In vitro experiments were performed at 5 °C in triplicate for each sample. The samples taken were analyzed by HPLC as above.

Prepared PLGA microparticles contained 451.38 µg of Alarelin per 100 mg of sample (encapsulation efficiency of 43.32%). The release kinetics of prepared PLGA microparticles in agar gel for initial 168 h is shown in Figure 1. Within 72 h, Alarelin was released with almost regular increments per 24 h (51.1 µg/24 h; 90.90 µg/48 h; 123.31 µg/72 h). The sample was treated as a delivery system with 1.2 µg of Alarelin released/mg of PLGA microparticles/72 h.

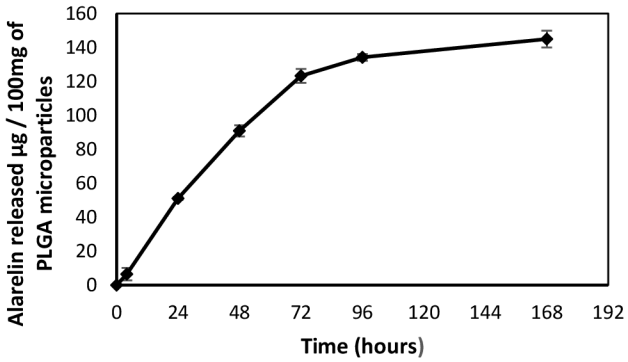


Figure 1. Release kinetics of Alarelin from PLGA microparticles.

2.2.2. Treatments

Four groups of randomly selected sterlet males (10 per group) received a single intramuscular injection of one of four preparations suspended in saline solution (0.9% NaCl, Braun Melsungen AG, Melsungen, Germany) (Table 1).

Table 1. Experimental groups of sterlet males.

Treatment	Fish Weight (kg)	Dose
0.9% NaCl	1.57 ± 0.23	1 mL/kg
Carp pituitary extract	1.59 ± 0.22	4 mg/kg
PLGA35 [1]	1.59 ± 0.25	35 µg/kg Alarelin
PLGA200 [1]	1.58 ± 0.27	200 µg/kg Alarelin

[1] PLGA microparticle delivery system with sustained Alarelin release.

2.3. Sample Collection

2.3.1. Blood Collection

A heparinized 5 mL syringe with a heparinized 21-gauge needle was used to collect serial blood samples (1000 µL) by caudal venipuncture before injection (0 h), and at 24, 48, and 72 h post-injection. Blood samples were centrifuged at 4000× *g* for 10 min at 8 °C, and plasma was stored at −80 °C until analysis.

2.3.2. Milt Collection

Milt was collected 24, 48, and 72 h post-treatment prior to blood collection. During the trial, fish were held at a constant temperature of 15 °C and natural illumination. Milt was collected by catheter from the urogenital duct into dry 50 mL plastic containers of known mass, avoiding contamination with feces or water. During collection, the male abdomen was gently massaged, allowing the complete release of milt from both Wolffian ducts. Milt from individual males was stored on ice at 4 °C for no longer than two hours during motility analysis.

2.4. Analysis of Samples

2.4.1. 11-Ketotestosterone and Testosterone Analysis

The commercially available enzyme-linked immunosorbent assay kits were used to determine plasma levels of testosterone (T; KAPD1559; DIAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium) and 11-ketotestosterone (11-KT; 582751; Cayman Chemical, MI, USA), according to the manufacturer's instructions, with each standard and plasma sample run in duplicate. The intra-assay coefficients of variation for T and 11-KT, calculated from the sample duplicates, were less than 6% in all tests, and inter-assay coefficients of variation were less than 7% for T and 11-KT. The absorbance of all assays was read with

a PlateReader AF2200 microplate reader (Eppendorf Czech and Slovakia s.r.o., Říčany u Prahy, Czech Republic).

2.4.2. Sperm Quantitative Parameters

Spermatozoon concentration of each sample was estimated using a Burker cell hemocytometer (Meopta, Prerov, Czech Republic) at 200× magnification on an Olympus BX 50 phase-contrast microscope (Olympus Czech Group, Prague, Czech Republic). Each of the containers containing collected milt was individually weighted to 10 mg accuracy, and mass of milt was used as a proxy of milt volume. Sperm production was estimated by index of relative sperm production (RSP, 10^9 spz/kg), computed as spermatozoon concentration multiplied by the volume of each sperm sample divided by the body weight of the corresponding male.

2.4.3. Sperm Qualitative Parameters

After sperm collection, spermatozoon motility parameters were evaluated for each male. Motility of sperm samples was initiated in 10 mM Tris-HCl solution, pH 8.0, containing 0.125% Pluronic F-127 (catalogue number P2443, Sigma-Aldrich) to avoid sperm sticking to the glass slide. Motility was recorded at 50 frames per sec by optical negative phase-contrast microscopy, at ×10 magnification objective (PROISER, Madrid, Spain), and IDS digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) for the first 100 s post-activation. Videos were analyzed to obtain kinetic data of spermatozoon motility with a 5 s interval starting at 10 s post-activation using the CASA plugin for ImageJ [20]. CASA analysis included the percent of motile cells, curvilinear velocity (VCL) in μm/s, and linearity (LIN) as ratio of velocity straight line to velocity average path (VSL/VAP). The cut-off for motile spermatozoa was set at VCL = 10 μm/s. Percent motility was determined at 10 s post-activation.

2.5. Statistical Analysis

2.5.1. Spermiation Rate

A χ^2 test was used to compare spermiation rate among the experimental groups.

2.5.2. Quantitative Sperm Parameters and Androgen Concentrations

As data were not normally distributed and showed significant difference in dispersion values (Kolmogorov–Smirnov and Levene's tests, respectively, $p < 0.05$), a nonparametric Kruskal–Wallis test was applied, followed by multiple comparisons of mean ranks for all groups. Tests were applied separately to compare groups at different times post-injection, and at the same time post-injection. The Mann–Whitney U-test was used for pairwise comparisons. An χ^2 test was used to compare spermiation rate among the experimental groups.

2.5.3. Sperm Qualitative Parameters

The sperm motility percentage, VCL, and LIN values for each combination of fish/experimental group/sampling time were extracted from the CASA dataset. Mean data of individual fish for each experimental condition were used to plot trend lines of VCL at 10–100 s post-activation time. Quadratic polynomial regression was selected for visualization of parameter trends. Before analysis, motility percentage and VCL data were tested for normality and homogeneity of variance by Kolmogorov–Smirnov and Levene's tests, respectively. All studied parameters were normally distributed and exhibited similar dispersion values. Data were analyzed by one-way ANOVA followed by Tukey's test.

Data of motility rate were transformed by the BOX-COX procedure before statistical analysis to obtain equality of variance among groups and analyzed first by two-way ANOVA with treatment as three levels (CP, PLG35, and PLG200), and post-injection time of three levels (24, 48, and 72 h). Tukey's test was used to assess differences among mean values of spermatozoon motility percentage in experimental groups.

Statistical analysis and graph plotting was performed using Statistica v. 13.5.0.17. (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Androgen Concentrations

3.1.1. Testosterone Concentrations

No differences in T concentration were observed among experimental groups at 0 h, 48 h, and 72 h, with the only intergroup differences between the PLGA and control groups observed at 24 h post-treatment. Administration of hormone treatment induced a significant increase in T values at 24 h compared to initial values, and declined thereafter (Figure 2).

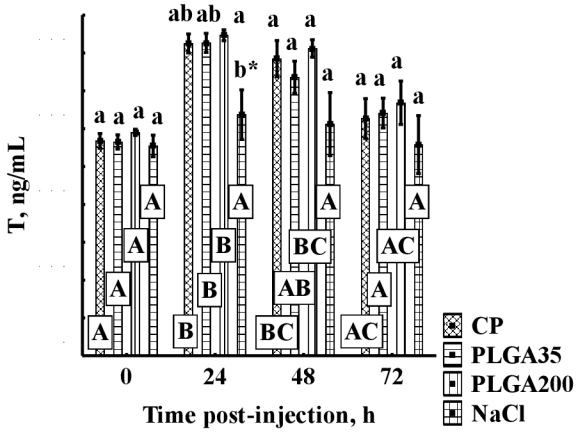


Figure 2. Testosterone concentration after hormonal treatments in sterlet. Significant differences among groups at a sampling point are indicated by lower case letters ($p < 0.05$). Significant differences within an experimental group at a different post-injection time are indicated by upper case letters (multiple comparisons of mean ranks for all groups, $p < 0.05$). *—indicates a significant pairwise difference between treatment by NaCl (control, dose 1 mL (0.9%NaCl)/kg) and each experimental group at 24 h post-injection (Mann–Whitney U-test, $p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg.

3.1.2. 11-KT Concentrations

No differences in 11-KT concentration were observed among experimental groups at 0 h and 24 h. No significant differences were detected between PLGA35 and PLGA200 groups at any sampling point. Administration of PLGA35 did not stimulate values of 11-KT significantly different from those detected in the control or CP group at any sampling point. PLGA200 induced higher values than NaCl at 48 h and 72 h and higher levels than CP at 48 h post-treatment. CP was associated with higher values than the control only at 72 h post-treatment (Figure 3).

Application of PLGA induced 11-KT values at 48 h and 72 h post-treatment significantly higher than initial values. Groups receiving CP showed a significant increase from initial values 72 h post-treatment (Figure 3).

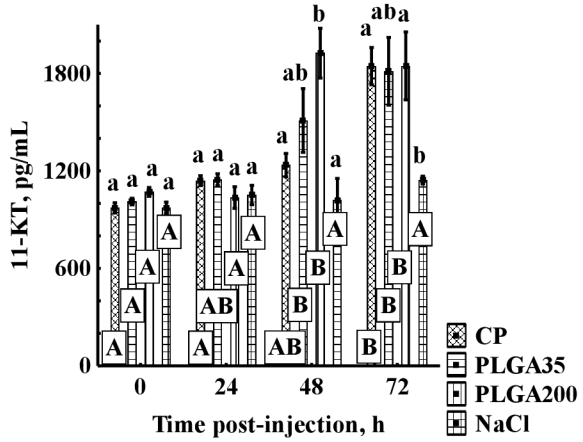


Figure 3. 11-KT concentration with and without hormone treatment in sterlet. Significant differences among groups at a sampling point are indicated by lower case letters ($p < 0.05$). Significant differences within an experimental group are indicated by upper case letters ($p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg; NaCl—control, dose 1 mL (0.9% NaCl)/kg.

3.2. Effect of Hormone Treatment on Sperm Production

3.2.1. Spermiation Rate

Spermiation was observed in six males in the CP group at 24 and 48 h post-injection, nine males in the PLGA35 group at 24, 48, and 72 h, and eight males in the PLGA200 group at 24, 48, and 72 h. No sperm was obtained from fish injected with saline solution. No significant differences were found among hormone-treated groups in the proportion of fish showing spermiation, and all treated groups differed significantly from the control group ($p < 0.05$).

3.2.2. Sperm Quantitative Parameters

Milt Volume

No significant differences in milt volume were observed among experimental groups 24 h post-treatment. At 48 h post-treatment, the PLGA200 group produced a significantly higher volume of sperm than seen with CP. A significant decrease in milt volume was observed in the group stimulated by CP at the second sampling time (48 h), with no spermiation detected at 72 h. Administration of PLGA treatments led to consistent volume throughout the trial period with no differences within or among groups (Figure 4).

Spermatozoon Concentration

CP initially induced high spermatozoon concentration 24 h post-treatment followed by a significant decrease at 48 h. Concentration in the PLGA35 group reached highest levels at 72 h, but did not differ from other groups at any sampling point. The PLGA200 group exhibited lower initial sperm concentration than with CP, but did not differ from PLGA35. Significantly higher values for PLGA200 were observed at 48 h and 72 h post-treatment compared to values at 0 h, although not differing at 48 and 72 h from other groups (Figure 5).

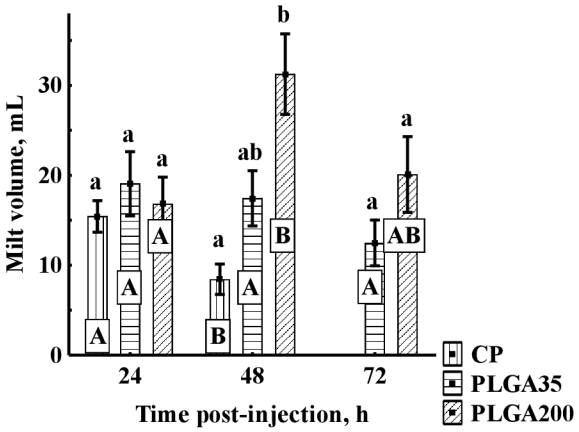


Figure 4. Milt volume after hormone treatment. Significant differences among groups at a sampling point are indicated by lowercase letters ($p < 0.05$). Significant differences within an experimental group are indicated by uppercase letters ($p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg.

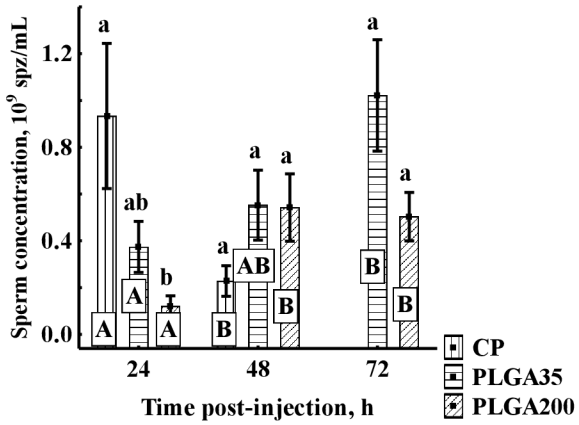


Figure 5. Spermatozoon concentration after hormone treatment. Significant differences among groups at a sampling point are indicated by lowercase letters ($p < 0.05$). Significant differences within an experimental group are indicated by uppercase letters ($p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg.

Relative Sperm Production

Similar to the trend in spermatozoon concentration, the administration of CP led to high RSP at 24 h post-treatment followed by a significant decline at 48 h, significantly differing at all points from PLGA200 but not from PLGA35. The PLGA35 group showed consistent RSP throughout the experiment, and did not differ from other groups at any sampling point. Application of PLGA200 was associated with lower initial RSP with significant increase at 48 h and 72 h (Figure 6).

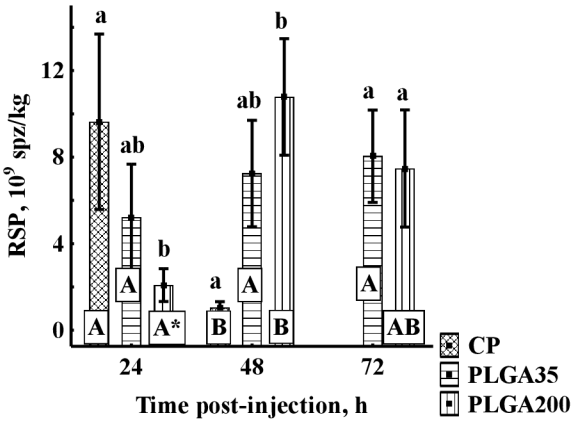
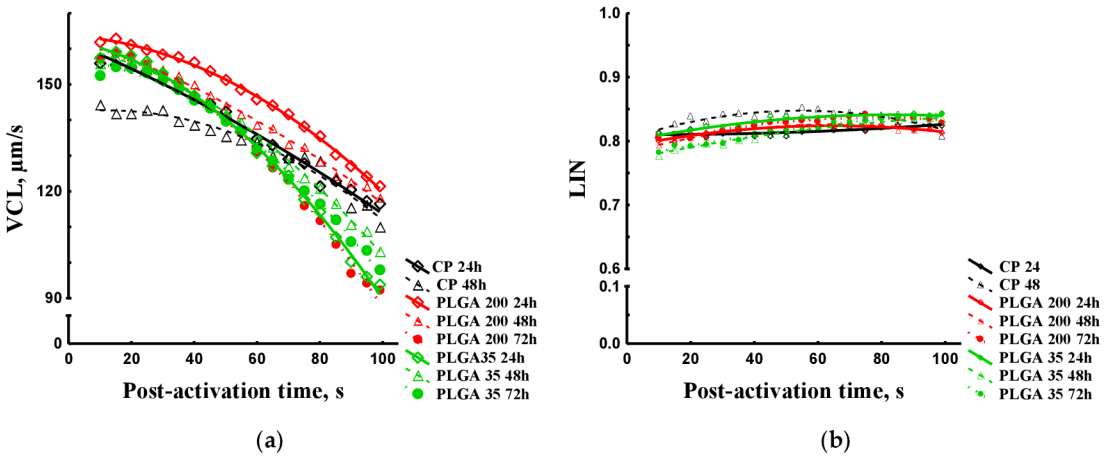


Figure 6. Relative sperm production (RSP) following hormone treatment. Significant differences among groups at a sampling point are indicated by lowercase letters ($p < 0.05$). Significant differences within an experimental group are indicated by uppercase letters ($p < 0.05$). *—indicates a significant pairwise difference between groups treated by PLGA 200 at 24 h in comparison to groups treated by PLGA200 at 48 h and 72 h post-injection (Mann–Whitney U-test, $p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg.

3.2.3. Sperm Qualitative Parameters

Spermatozoon Curvilinear Velocity and Linearity

After spermatozoon motility activation, VCL decreased in all experimental groups (Figure 7a) with no differences among groups. The dynamics of mean LIN values in experimental groups did not allow selection of points of interest, as values were similar, and no significant differences were found.



(**a**) (**b**)

Figure 7. Kinematic parameters of sterlet spermatozoa obtained after hormone treatments. (**a**) VCL dynamics post-activation. (**b**) LIN dynamics post-activation. Data are presented as mean (dots) and quadric polynomial regression lines. Abbreviations: CP 24 h, CP 48 h-treatment by carp pituitary extract, dose 4 mg/kg, samples collected at 24 and 48 h post-injection respectively; PLGA 200 24 h, PLGA 200 48 h, PLGA 200 72 h—treatment by Alarelin, dose 200 µg/kg, samples collected at 24, 48, and 72 h post-injection respectively; PLGA 35 24 h, PLGA 35 48 h, PLGA 35 72 h—treatment by Alarelin, dose 35 µg/kg, samples collected at 24, 48, and 72 h post-injection respectively.

Spermatozoon Motility Rate

Two-way ANOVA applied to motility rate revealed a significant effect of treatment ($p < 0.001$). In contrast, effects of time post-injection and the interaction of these two factors were not significant ($p = 0.989$ and $p = 0.751$, respectively). Tukey's test was used to compare mean values of spermatozoon motility rate in experimental groups without consideration of time post-injection. Motility rate was significantly lower in the PLGA200 group than in PLGA35, while no differences were found between CP and the PLGA groups (Figure 8).

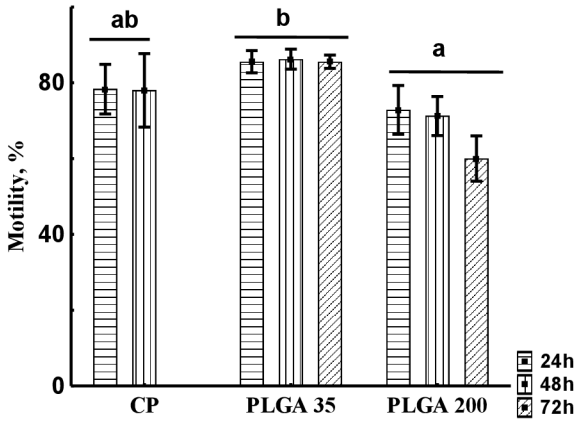


Figure 8. Sperm motility percentage (Motility, 10 s post-activation) of sterlet sperm samples obtained after hormonal treatments at different post-injection times (24, 48, and 72 h). Horizontal Lines indicate no significant f factor "post-injection time" (two-way ANOVA, $p = 0.989$). Different letters indicate significant differences among treatments by different hormones, combined by the factor "post-injection time" (two-way ANOVA, $p < 0.001$; Tukey's post-hoc test, $p < 0.05$). Experimental treatments: CP—carp pituitary extract, dose 4 mg/kg; PLGA35—Alarelin, dose 35 µg/kg; PLGA200—Alarelin, dose 200 µg/kg.

4. Discussion

Hormone treatment is a prerequisite for successful spermiation in cultured sterlet males [19]. In the current study, a PLGA microparticle system with continuous Alarelin release significantly prolonged the spermiation period and increased the number of expressible spermatozoa, as compared to standard treatment by carp pituitary suspension.

The spermiation-stimulating effect of CP suspension has been known for decades, and it is widely used in sturgeon aquaculture [9]. The CP directly stimulates testicular steroidogenesis and does not rely on endogenous LH stores in the pituitary [5].

Administration of CP in our trial stimulated initiation of spermiation with maximum RSP 24 h post-treatment followed by a significant decrease at 48 h and no expressible sperm at 72 h post-treatment. A time curve of spermiation decline after CP treatment similar to our findings has been previously reported in sterlet [21] and in paddlefish [22]. The spermiation-inducing effect of gonadotropin preparations is usually more rapid and shorter-lasting than GnRHa treatment [4,22]. Analogues of GnRH act at a higher level of the hypothalamic-pituitary-gonad axis, and induce secretion of endogenous LH and possibly other factors that may be involved in the regulation of spermiation [23]. In sterlet, the administration of GnRHa alone dissolved in saline solution has not been shown to initiate satisfactory sperm production [19,24]. The low efficacy of a single GnRHa injection in inducing spermiation in sterlet is probably a combined result of the short residence time of GnRHa in circulation [25] and dopamine inhibition of LH secretion [19,24]. Co-administration of GnRHa with a dopamine antagonist led to a significant improvement of spermiation parameters compared to GnRHa alone [24]. Combined treatment of GnRHa

and the dopamine antagonist used by Alavi et al. [4] stimulated maximal sperm production 48 h post treatment, followed by a significant decline at 72 h post treatment.

One of the advantages of the GnRHa peptide is its low molecular weight and efficacy in μg doses, enabling its incorporation into delivery systems with release over a prolonged period [8]. The controlled delivery systems of GnRHa have proven to be effective stimulators of spermiation in several marine species [11]. Mentioned results with marine species are in agreement with our observation in sterlet, where PLGA microparticle system with continuous Alarelin release enabled production of high-quality sperm over 72 h with no differences between PLGA35 and CP at 24 and 48 h post-treatment. Interestingly the treatment PLGA200 (200 μg kg^{-1}) was associated with a significantly lower RSP than the CP group at 24 h, but no difference was found in milt volume.

In contrast to our results, unsatisfactory spermiation using slow-release EVAc was reported by Alavi et al. [4]. This may have resulted from the release kinetics of Alarelin from PLGA microparticles compared to EVAc implants. Release of Alarelin from PLGA microparticles is characterized by an immediate initial burst and a sustained or continuous decline until depletion of the microspheres (Figure 1). Significant portion of Alarelin incorporated in our delivery systems was released within 72 h post-injection. The Alarelin at 35 μg kg^{-1} induced adequate spermiation at all sampling points and outperformed the 200 μg kg^{-1} treatment in spermatozoon motility. Experiments in other fish species have confirmed that unnecessarily high doses of GnRHa can negatively influence final gamete maturation [26,27]. No differences were detected between sperm samples obtained after treatment by CP and PLGA delivery systems in term of velocity or linearity parameters.

Final sperm maturation is a crucial component of the life cycle of fish, being under the control of the LH stimulating production of sex steroids [28]. Androgens T and 11-KT are the predominant sex steroids in male teleost fish [29]. In the current study, a positive correlation between T and 11-KT was observed, with all the hormone treatments significantly increasing T and 11-KT values, a decline in T at the end of the trial, and remaining high values of 11-KT. This observation is consistent with the fact that T acts as a precursor of 11-KT, with 11-KT playing an important role in the initiation of spermiation [30].

The efficacy of the PLGA microparticle delivery system compared to acute application of GnRHa in saline solution demonstrates the ability of sustained Alarelin release to stimulate satisfactory spermiation results, despite the assumed dopamine inhibition of LH secretion in sterlet males [19,24], and induce favorable results both quantitively and qualitatively. The elimination of a dopamine antagonist in spermiation treatment of sterlet is desirable, considering its long half-life and wide range of potential side effects.

5. Conclusions

A PLGA microparticle system with sustained release of Alarelin at 35 μg kg^{-1} effectively induces spermiation in sterlet, significantly outperforming CP in prolonged stimulation of spermiation.

Author Contributions: Conceptualization, P.P. and B.D.; methodology, P.P., B.D., J.V., J.K. and K.K.; software, S.B.; validation, V.K. and S.B.; formal analysis, B.D. and P.P.; investigation, P.P., B.D., S.B., V.K., J.K., A.S. and M.R.; resources, P.P., M.R. and D.G.; data curation, B.D.; writing—original draft preparation, P.P.; writing—review and editing, B.D.; visualization, P.P. and B.D.; supervision, B.D., P.P. and J.V.; project administration, P.P.; funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Agriculture of the Czech Republic, grant number NAZV QK1810221.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and followed national and international guidelines for the protection of animal welfare (EU-harmonized Animal Welfare Act of the Czech Republic). The experimental unit is licensed (No. 2293/2015-MZE-17214 and No. 55187/2016-MZE-17214 within project

NAZV QK1810221 according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors give special thanks to Ing. Pavel Šablatura for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 7

THE EFFECT OF HORMONAL TREATMENT ON SELECTED SPERM QUALITY PARAMETERS AND SEX STEROIDS IN TROPICAL CYPRINID BALA SHARK *BALANTIOCHEILOS MELANOPTERUS*

Podhorec, P., Knowles, J., Vysloužil, J., Boryshpolets, S., Sotnikov, A., Holická, M., Kouřil, J., Dzyuba, B., 2022. The effect of hormonal treatment on selected sperm quality parameters and sex steroids in tropical cyprinid bala shark *Balantiocheilos melanopterus*. Fishes 7, 122.

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The Effect of Hormonal Treatment on Selected Sperm Quality Parameters and Sex Steroids in Tropical Cyprinid Bala Shark *Balantiocheilos melanopterus*

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Simple Summary: The bala shark *Balantiocheilos melanopterus* does not reproduce naturally under culture conditions, and hormone stimulation is routinely administered to male broodstock to ensure production of adequate quantities of high-quality sperm. Poly(lactic-co-glycolic acid) (PLGA) microparticles with slow release of 10 μg/kg fish body weight GnRHa were injected to induce spermiation in bala sharks and to compare them with standard treatments in tropical cyprinid culture: recombinant hCG and GnRHa with metoclopramide. The PLGA microparticle treatment led to significantly higher plasma T and 11-KT concentrations at 24 h post-injection than was detected in the control group. Sperm motility parameters were evaluated during the 10–60 s after motility activation at 2 s intervals. Starting from 28 s post-activation, the motility percentage was significantly higher in the PLGA group compared to the control group. The PLGA microparticle treatment was also found to significantly increase sperm volume and total sperm count compared to control (0.9% NaCl). The PLGA microparticle system with continuous release of GnRHa was identified as optimal for inducing spermiation in cyprinid bala sharks.

Abstract: Hormone treatments are routinely employed with bala shark *Balantiocheilos melanopterus* male broodstock to stimulate the production of high-quality sperm. In the current trial, three spermiation-inducing treatments were evaluated: 20 μg/kg body weight (BW) recombinant hCG; 20 μg/kg BW [D-Ala^6, Pro^9, NEt]- gonadotropin-releasing hormone (GnRHa) with 20 mg/kg BW metoclopramide; and poly(lactic-co-glycolic acid) (PLGA) microparticles with slow release of 10 μg/kg BW GnRHa. A 0.9% saline AS negative control was also included. Administration of the GnRHa through the form of slow release of PLGA microparticles 24 h after treatment resulted in a significantly higher sperm volume, motility percentage, and total sperm count compared to the control. Injection of GnRHa with metoclopramide induced sperm parameters that did not differ from the control, with the exception of motility percentage. The lowest potency to induce spermiation in bala sharks was in the treatment with recombinant hCG. Both PLGA microparticles and GnRHa with metoclopramide significantly increased blood plasma concentrations of testosterone and 11-ketotestosterone compared to the control. The PLGA microparticle system with continuous release of 10 μg/kg BW GnRHa was the most effective treatment in inducing spermiation in bala sharks.

Keywords: sperm; cyprinid; sustained release; reproduction; luteinizing hormone

Citation: Podhorec, P.; Knowles, J.; Vysloužil, J.; Boryshpolets, S.; Sotnikov, A.; Holická, M.; Kouřil, J.; Dzyuba, B. The Effect of Hormonal Treatment on Selected Sperm Quality Parameters and Sex Steroids in Tropical Cyprinid Bala Shark *Balantiocheilos melanopterus*. *Fishes* **2022**, *7*, 122. https://doi.org/10.3390/fishes7030122

Academic Editor: Adelino V. M. Canario

Received: 3 May 2022
Accepted: 26 May 2022
Published: 30 May 2022

1. Introduction

The bala shark *Balantiocheilos melanopterus* is an omnivorous and pelagic cyprinid species native to rivers and lakes of Sumatra and Borneo in Southeast Asia [1]. During the rainy season, the bala shark migrates to reach specific breeding grounds where it undergoes mass spawning [2]. It is a popular aquarium species, with natural populations currently declining in much of its native range because of habitat degradation and overfishing for the aquarium trade [3]. It is listed as vulnerable on the IUCN Red List of Threatened Species, with only cultured specimens available for the pet trade.

Bala sharks do not reproduce spontaneously under culture conditions, and hormone therapy is necessary to stimulate final oocyte maturation and ovulation [4]. Although bala shark males usually complete spermatogenesis and spermiation in captivity, the quality of sperm obtained is variable. Hormone treatments are routinely employed to ensure an adequate quantity of high-quality sperm. Hormone treatments can be categorized, according to mode of action, as gonadotropin preparations, e.g., human chorionic gonadotropin (hCG); treatments involving the carp pituitary acting at the level of the gonad [5]; and gonadotropin-releasing hormone analogues (GnRHa) with or without dopamine antagonists, acting on the pituitary and hypothalamus [6]. GnRHa is a small peptide of low molecular weight that can be administered in sustained-release delivery systems [7]. In contrast to the situation in most mammals, extended exposure of fish organisms to exogenous GnRHa stimulation does not cause desensitization of the pituitary gonadotrophs in fish, but elevates levels of plasma luteinizing hormone (LH) and stimulates the natural progression of steroidogenesis associated with spermiation [8].

Common sustained-release systems in aquaculture include ethylene-vinyl acetate copolymer (EVAc) implants [9], solid implantable cholesterol pellets [10], and poly(lactic-co-glycolic acid) (PLGA) microparticles [11]. Poly(lactic-co-glycolic acid) is biodegradable, has excellent biocompatibility, and is safe for use in pharmaceutical products [12]. Encapsulated GnRHa is released via degradation and erosion of the polymer matrix [13], leading to prolonged stimulation of LH [8]. The PLGA microparticles have the advantage that they are administered as a liquid suspension, enabling fish-specific dosing based on body weight. Treatment with GnRHa through sustained-release systems is reported to significantly increase the quantity and quality of expressible milt in several fish species [14–16]. Most trials of sustained-release systems have focused on marine species [8], with the few studies in freshwater aquaculture producing suboptimal results [10,17,18].

An essential prerequisite of successful reproduction in captivity is synchronization of maximum sperm production with ovulation and collection of ripe eggs. This is especially crucial in bala shark, as numbers of available male broodstock are usually low, and the species' low tolerance to manipulation and handling may have fatal consequences (unpublished observation).

The goal of the current study was to assess the efficacy of sustained-release GnRHa from PLGA microparticles compared to the single-dose treatments commonly used in tropical cyprinids (recombinant hCG and GnRHa with dopamine antagonist) in stimulating spermiation in bala sharks. Evaluation of the experimental treatments was based on the qualitative and quantitative characteristics of the obtained sperm and plasma sex steroid profiles.

2. Materials and Methods

2.1. Animals and Maintenance

Subadult bala shark broodstock (two years old) were purchased in 2018 from a local ornamental fish importer and held for one year in an aquaculture system comprising separate 600 L tanks (dimensions: 120 cm × 100 cm × 50 cm) with mechanical and biological filtration systems at the University of South Bohemia in Ceske Budejovice. The photoperiod was set to 12:12 L:D. Tap water (after dechlorination by activated carbon) with the following characteristics was used (mean ± SD): pH 8.2 ± 1.9, electrical conductivity 290 ± 35 µS/cm at 25 °C, temperature 26.0 ± 0.5 °C, and dissolved oxygen 7.8 ± 0.4 mg/L. Every seven days, 50% of the water was changed. Fish were fed twice daily to apparent

satiation using commercial extruded feed for ornamental fish (Tetra Discus; Tetra GmbH, Melle, Germany), with a daily addition of frozen bloodworms. After one year, males averaging 180 ± 40 g body weight (BW) were randomly divided into four groups of 10 and experimental treatments were applied. No significant differences in BW were found among experimental groups using one-way ANOVA ($p < 0.05$).

2.2. Hormone Treatments

2.2.1. Preparation of PLGA Microparticles with Continuous GnRHa Release

The microparticles were prepared by solvent evaporation from a multiple water/oil/water emulsion. During evaporation, solid spherical particles are formed from the polymer, which entraps the drug in its structure. Alarelin (APExBIO, Houston, TX, USA), a GnRH analogue, was used as a hormonally active substance. A copolymer of 75% polylactic acid and 25% polyglycolic acid (PLGA) was used as carrier material (Resomer 753, Evonik, Darmstadt, Germany).

For the oil phase, 800 mg of PLGA of the appropriate Resomer and 5 g of dichloromethane (Penta, Prague, Czech Republic) were weighed into a tube and allowed to dissolve. The inner aqueous phase was prepared by dissolving gelatin in purified water at 65 °C (10%) (Sigma Aldrich, St. Louis, MO, USA). The external aqueous phase consisted of two solutions: a premixed 1% polyvinyl alcohol (PVA) (Sigma Aldrich, St. Louis, MO, USA) and the main continuous aqueous phase with 0.1% PVA.

The next step consisted of preparation and further processing of the emulsion from the individual phases. On an analytical balance, 10 mg of GnRHa was weighed into a microcentrifuge tube, and 1.5 mL of a 10% gelatin solution was added using a syringe. The microcentrifuge tube with prepared internal aqueous phase was vortexed to dissolve the drug. The resulting solution was then poured into the wide-necked tube containing the PLGA/dichloromethane oil phase (800 mg/5 g) and vortexed for 30 s to ensure primary emulsification through the formation of a crude primary water/oil emulsion. The emulsion was then mixed for 1 min using a homogenizer (T25 basic, IKA-Werke, Staufen, Germany), converting the dispersed phase to smaller droplet sizes and producing a fine emulsion. Subsequently, 12 g of a 1% PVA solution was added to a wide-necked tube and homogenized for 1 min to produce a concentrated multiple water1/oil/water2 emulsion. The emulsion was then added to a larger beaker with 200 mL of a 0.1% PVA solution with 2% NaCl for dilution, and the contents were stirred for two hours using a mechanical stirrer at 450 rpm. During this time, the organic solvent evaporated, and the polymer solidified into spherical particles. The resulting micro-suspension was filtered through a 250 µm screen for the eventual separation of agglomerates. Isolation of the microparticles was performed by continuous centrifugation (3461× *g* for 2 min). Excess water was decanted, and the microparticles were collected in Petri dishes, stored in a freezer, and dried by lyophilization.

The alarelin content in PLGA microparticles was determined using HPLC. The PLGA microparticles were dissolved in acetone. The resulting solution was mixed 1:1 (*v*/*v*) with a phosphate buffer of pH 7.0 (Fisher Scientific, spol. s.r.o., Pardubice, Czech Republic). The mixture was filtered through a 0.45 µm membrane filter. The content was quantified by HPLC (Agilent 1100, Agilent, Santa Clara, CA, USA) with a NUCLEODUR 100-5 CN-RP (150 mm × 4.6 mm, 5 µm) column. A binary mixture of acetonitrile and 20 mM H_3PO_4 (16:84, *v*/*v*) was used as a mobile phase with a flow rate of 0.8 mL/min, with the temperature set at 30 °C, an injected sample volume of 20 µL, and a 220 nm detection wavelength.

For the drug-release study, 50 mg of microparticles was suspended in 1%/0.4 mL agarose solution in a glass vial and cooled to solidify the agarose, then 800 µL agarose was added as covering layer. When the agar had solidified, 5 mL phosphate buffer was added. Precisely 2 mL of the buffer was collected after 4, 24, 48, 72, 96, and 168 h and filtered through a 0.22 µm membrane filter.

After each sampling, the buffer residue in vials was removed, the vials were washed with 0.5 mL buffer, and 5 mL of fresh buffer was added. In vitro experiments were run at

5 °C and performed in triplicate for each sample. Collected samples were analyzed using the HPLC method as described.

Prepared PLGA microparticles contained 45 µg of Alarelin per 100 mg of sample (encapsulation efficiency of 43%). The release kinetics in Figure 1 show, that, over the course of seven days, approximately one-third of the drug was released (144.75 µg per 100 mg of the sample). However, the main portion of the release took place during first 72 h. During this time, the drug was released with almost regular increments per 24 h (51.1 µg/24 h; 90.90 µg/48 h; 123.31 µg/72 h), very similar to advantageous zero-order kinetics. The sample was treated as a delivery system with 1.2 µg of Alarelin released/mg of PLGA microparticles/72 h.

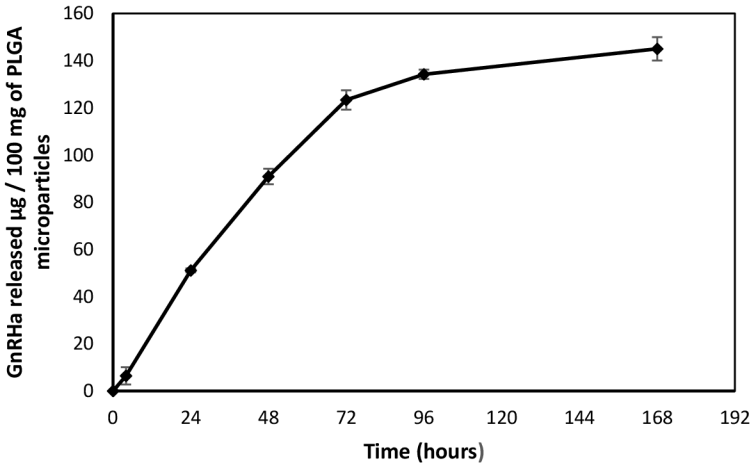

Figure 1. Kinetics of release of GnRHa from PLGA microparticles.

2.2.2. Hormone Treatments

To evaluate the effectiveness of PLGA microparticles with GnRHa in stimulating bala shark sperm release, comparisons were made with both negative (0.9% NaCl) and positive controls. As positive control groups, commonly used treatments based on GnRHa with dopamine antagonist and recombinant hCG were selected. The doses were established based on previous successful trials with cyprinids for combined treatment [19–21] and for hCG treatment [22,23]. In our recently published study, we have shown excellent tolerance in carp to the PLGA microparticle doses used in current study [24].

Male fish were administered a single intramuscular injection of:

(1) 1 mL/kg BW 0.9% NaCl (Braun Melsungen AG, Melsungen, Germany); control group.
(2) 20 µg/kg BW recombinant hCG (Ovitrelle, Merck Europe B.V., Amsterdam, The Netherland); hCG group.
(3) 25 µg/kg BW [D-Ala^6, Pro^9, NEt]-GnRH (APExBIO, Houston, TX, USA) combined with 20 mg/kg BW metoclopramide (Met) (Sigma-Aldrich, St. Louis, MO, USA); GnRHa + Met group.
(4) 10 µg/kg BW [D-Ala^6, Pro^9, NEt]-GnRH in PLGA microparticles; PLGA group.

All substances were dissolved in 0.9% NaCl.

2.3. Collection and Analysis of Samples

2.3.1. Blood and Sperm Sample Collection

Heparinized needles in 1 mL syringes were used to collect blood samples (400 µL) by caudal venipuncture before injection and 24 h post-injection (PI). Blood samples were centrifuged at 4000× *g* for 10 min at 8 °C, and plasma was stored at −80 °C until analysis. Fish were anaesthetized with 0.03 mL/L clove oil before manipulation.

Sperm was collected once at 24 h post-treatment, as attempts at multiple sperm collection led to health problems and mortality. The urogenital pore was dried with a paper towel before direct sperm collection. To counteract urine contamination, sperm was collected into 5 mL syringes containing 2.0 mL of Kurokura 180 immobilizing solution [25]. After measuring total sperm volume, syringes were immediately placed on ice and transported to the laboratory for analysis. Sperm samples from individual males were stored on ice at 4 °C for not longer than two hours during motility analysis.

2.3.2. Testosterone and 11-Ketotestosterone Analysis

Plasma levels of testosterone (T; KAPD1559; DIAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium) and 11-ketotestosterone (11-KT; 582751; Cayman Chemical, MI, USA) were evaluated by ELISA using commercially available kits according to the manufacturer's instructions, with each standard and plasma sample run in duplicate. The intra-assay coefficients of variation for T and 11-KT were less than 6% in all tests, and inter-assay coefficients of variation were less than 7% for T and 11-KT. The absorbance of all assays was read by a PlateReader AF2200 microplate reader (Eppendorf Czech and Slovakia s.r.o., Říčany u Prahy, Czech Republic).

2.3.3. Sperm Production Indexes

The volume of sperm was estimated by measurement of sperm sample mass to the nearest 10 mg. The sperm concentration of each sample was estimated using a Burker cell hemocytometer (Meopta, Prerov, Czech Republic) at 200× magnification on an Olympus BX 50 phase-contrast microscope (Olympus Czech Group, Prague, Czech Republic). Total sperm count was computed as sperm concentration multiplied by the volume of the sperm sample. Normalized-by-male-BW sperm volume (mL/kg) and sperm count (TSP, 10^{10} spz/kg) were presented as sperm production indexes.

2.3.4. Sperm Motility Analysis

Sperm was activated in distilled water containing 0.125% Pluronic F-127 (catalogue number P2443, Sigma-Aldrich) to avoid sperm sticking to the glass slide. Motility records were made from the bottom part of the drop. Motility was recorded at 50 fps using optical negative phase-contrast microscopy, a ×10 magnification lens (PROISER, Madrid, Spain), and an IDS digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany). The total number of spermatozoa in which motility parameters were analyzed at each time point ranged from 910 to 2774. Altogether, 198,009 spermatozoa were analyzed. The videos were recorded for the first 60 s after motility activation, and kinetic data of sperm motility were collected at 2 s intervals beginning at 10 s post-activation using the CASA plugin for ImageJ (Purchase, Earle, 2012). Kinetic parameters obtained by CASA for all sperm samples used in the study were subjected to a correlation analysis using Spearman's rank correlation coefficient. To simply data presentation, only parameters with a low correlation coefficient ($r < 0.06$) were selected as descriptors of sperm motility. These parameters were the percentage of motile cells, curvilinear velocity (VCL) in μm/s and the linearity of track (LIN).

2.4. Statistical Analysis

2.4.1. Kinetic Parameter Analysis

Kinetic parameters were subjected to a correlation analysis using Spearman's rank correlation coefficient. All spermatozoa with VCL < 10 μm/s were considered immotile and excluded from the analysis. The percentage of motile spermatozoa and VCL and LIN values for each combination of male/experimental group/time post-activation were extracted from the CASA dataset. The mean motility rate for each male was used to plot trend lines for the motility rate at 10–60 s post-activation. Quadratic polynomial regression was selected for visualizing motility trends. These trend lines were used to determine the "time points of interest" at which the significance of differences among groups was additionally evaluated. Before analysis, the data were tested for normality

and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests, respectively. All studied parameters were normally distributed and had similar dispersion values; the data were first analyzed by two-way ANOVA. Factor "treatment" (four levels: control, hCG, GnRHa + Met, and PLGA) and "time" (25 time post-activation time points) were significant for VCL, LIN, and motility percentage at $p < 0.001$, and interaction of factors was insignificant for VCL and LIN (at $p > 0.1$) and significant for motility percent ($p < 0.01$). Tukey's test was used to quantify differences among treatments (mean values of the motility rate, VCL, and LIN in individual males) at each sampling time and between time points of interest for each experimental treatment.

2.4.2. Total Sperm Count

As data for the TSP were normally distributed and showed no significant differences in dispersion values (Kolmogorov–Smirnov and Levene's tests, respectively), parametric one-way ANOVA was applied, and Tukey's honest significant difference test was used to assess differences among groups.

2.4.3. Testosterone and 11-Ketotestosterone Analysis

Sex steroid values were not normally distributed and showed significant differences in dispersion values (Kolmogorov–Smirnov and Levene's tests, respectively, $p < 0.05$). The Kruskal–Wallis test was used to analyze differences among groups, followed by multiple comparisons of mean ranks for all groups. These tests were applied separately to compare the treatment groups at different times PI and at the same time PI.

Statistical analysis and graph plotting were performed using Statistica v. 13.5.0.17 (TIBCO Software Inc., Palo Alto, CA, USA). Null hypotheses were rejected at $p < 0.05$ in all applied statistical tests.

3. Results

3.1. Plasma Concentration of Testosterone and 11-Ketotestosterone

The PLGA sustained-release system and GnRHa + Met treatment led to significantly higher plasma T concentrations at 24 h PI than detected in the control group. Treatment with hCG did not elicit plasma T concentrations significantly different from the control group ($p < 0.05$) or other experimental groups (Figure 2a).

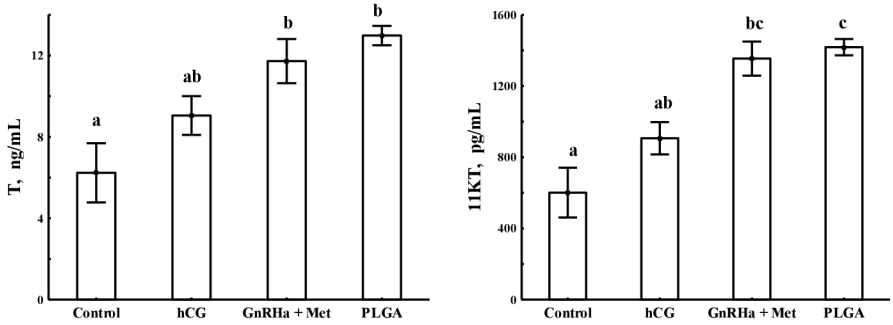

Figure 2. (**a**) Plasma testosterone (T) concentrations in bala sharks after hormone treatment; (**b**) plasma 11-ketotestosterone (11-KT) concentration after hormone treatment in bala sharks. Control (1 mL/kg 0.9% NaCl); hCG (20 μg/kg recombinant hCG); GnRHa + Met (25 μg/kg [D-Ala^6, Pro^9, NEt]-GnRH with 20 mg/kg metoclopramide); PLGA (PLGA microparticles). Different letters indicate significant differences ($p < 0.05$; Kruskal–Wallis test, multiple comparisons of mean ranks for all groups). Data are expressed as the mean ± SE.

The PLGA sustained-release system and GnRHa + Met treatment led to significantly higher plasma 11-KT concentrations at 24 h PI ($p < 0.05$) than detected in the control group. Treatment with hCG did not elicit plasma 11-KT concentrations significantly different from the control group ($p < 0.05$) or GnRHa + Met. Significantly lower 11-KT values were found after hCG treatment compared to the PLGA at 24 h PI (Figure 2b).

3.2. Sperm Production Indexes

All males in the trial produced viable sperm.

The PLGA group showed significantly higher normalized sperm volumes than the control and hCG group but did not differ from the group treated with GnRHa + Met. No differences were found among control, hCG, and GnRHa + Met groups in sperm volume ($p < 0.05$) (Figure 3a). No significant differences in sperm concentration were detected among experimental groups (Figure 3b).

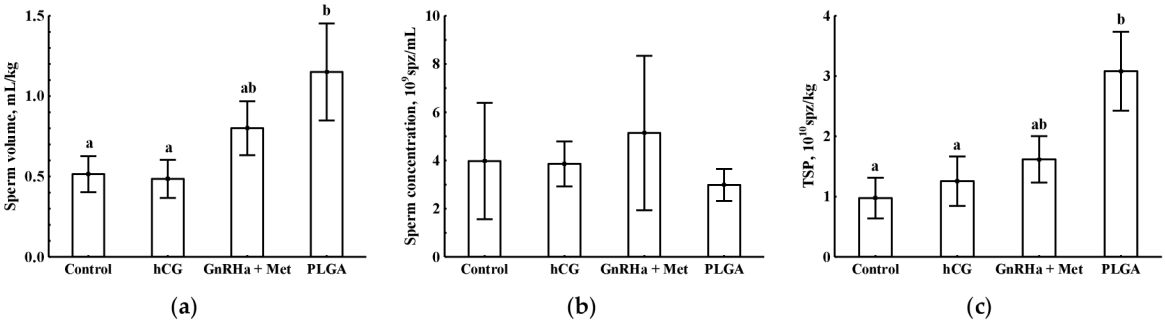

Figure 3. Sperm production indexes after hormonal treatment in bala sharks. (**a**) Normalized sperm volume; (**b**) sperm concentration; (**c**) normalized total sperm count after hormonal treatment in bala sharks. Control (1 mL/kg 0.9% NaCl); hCG (20 µg/kg recombinant hCG); GnRHa + Met (25 µg/kg [D-Ala^6, Pro^9, NEt]-GnRH with 20 mg/kg metoclopramide); PLGA (PLGA microparticles). Different letters indicate significant differences ($p < 0.05$; one-way ANOVA, Tukey's HSD test). Data are expressed as the mean ± SE.

The PLGA group showed significantly higher total sperm counts than the control group and the group treated with hCG but did not differ from the group treated with GnRHa + Met. No differences were found among control, hCG, and GnRHa + Met groups in total sperm counts ($p < 0.05$) (Figure 3c).

3.3. Sperm Motility Parameters

After sperm motility activation VCL, LIN, and motility percentage were dynamically changed in a treatment-specific way, and the obtained regression lines made it possible to determine the "points of interest" for the next step of the statistical analysis (Figure 4). Generally, the motility percentage and VCL decreased 18–20 s post-activation in all experimental groups.

However, at the initial stage of motility (10 s post-activation), motility percentage was significantly lower in the GnRHa + Met than in all other groups, while no significant differences in average VCL among experimental groups at this post-activation time point were found. Starting from 30 s post-activation, motility percentage was significantly higher in the PLGA group compared to the control group, and starting from 34 s post-activation, it was also higher in the PLGA group compared to both control and hCG groups, and these dependencies were the same until 60 s post-activation. A significant increase in motility percentage between 10 s and 22 s post-activation was observed in the GnRHa + Met group only, and no significant rise in VCL was found between 10 s and 20 s post-activation in all groups. A significant decrease in VCL (in comparison to 10 s post-activation) was observed after 20 s post-activation in the GnRHa + Met group and 28 s post-activation in the control

and hCG groups. Significantly lower LIN in the PLGA group in comparison to the hCG group was found at late stages of motility (38–60 s post-activation).

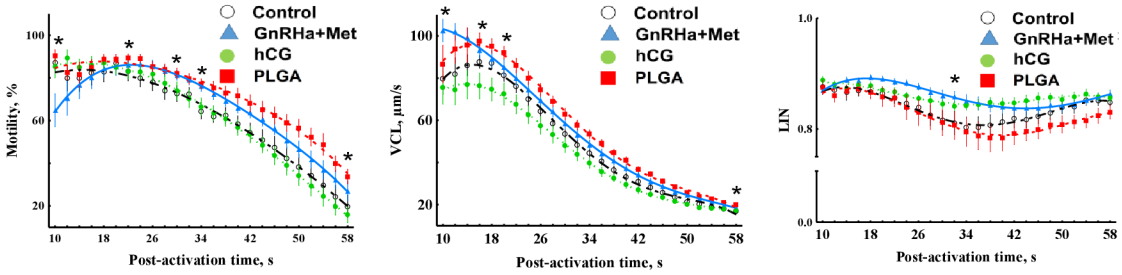

Figure 4. Dynamics of motility parameters during post-activation time for Bala shark spermatozoa obtained after hormonal treatments. (**a**) Sperm motility percentage (Motility); (**b**) curvilinear velocity (VCL); (**c**) sperm track linearity (LIN). Control (1 mL/kg 0.9% NaCl); hCG (20 μg/kg recombinant hCG); GnRHa + Met (25 μg/kg [D-Ala^6, Pro^9, NEt]-GnRH with 20 mg/kg metoclopramide); PLGA (PLGA microparticles). Data are presented as means (dots) ± SE (whiskers), lines are quartic polynomial regression lines, * indicates a "point of interest" discussed in the text.

4. Discussion

Injection of PLGA microparticles with continuous GnRHa release stimulated a significant increase in sperm volume and total sperm count, but not motility parameters, at the initial stage of motility in the sperm of bala shark males compared to a control group.

Similar results have been reported in marine fish species after administration of sustained-release systems, showing increased quantity [14–16] and quality [26] of expressible sperm. The use of sustained-release delivery systems in marine aquaculture is based on the need for prolonged stimulation of LH levels in species with asynchronous oocyte development and multiple spawning [8]. Administration of a single GnRHa injection is insufficient due to its short residence time in circulation [27]. On the other hand, in most commercially important freshwater cyprinids, a single injection of GnRHa with a dopamine antagonist is an effective inducer of an LH surge, leading to enhanced spermiation [19], and, in the trials performed so far, significantly outperforming sustained-release systems [17,18]. However, our data show that 24 h sustained release of GnRHa alone led to significantly higher sperm volumes, motility rates, and total sperm counts compared to untreated controls. In contrast, treatment with combined GnRHa and dopamine antagonist did not enhance sperm compared to the control group except for the motility rate.

The observed increase in bala shark sperm volume 24 h after administration of the sustained release system can be attributed to a stimulatory effect on seminal fluid production, which leads to hydration of testes [28] and acquisition of motility capacity in the existing intra-testicular sperm [5]. The increased fluid content of the testes allowed stripping of more sperm, which are present within the testes but otherwise would not be released. No decrease in sperm concentration was found after injection with the sustained-release system, despite significantly increased sperm volume compared to the control group.

Hormone induction does not usually influence sperm quality parameters, such as motility rate, motility duration, or sperm velocity, in captive species with moderate spermiation ability [16]. Improvement of sperm quality parameters is more characteristic of species with severe reproductive dysfunctions and little or no spermiation under captive conditions, such as sturgeon [18] and flatfishes [26]. All bala shark males in our trial completed spermiogenesis, and expressible milt could be obtained prior to treatment. In addition, as minor differences among the experimental groups in comparison to control were found in terms of motility percentage, VCL, and LIN, it can be summarized that hormonal treatment by PLGA led to production of greater amounts of sperm that could be collected but not to increased sperm quality parameters. In studies of freshwater and marine fishes [4,18,19],

significantly increased seminal plasma and sex steroid values linked to higher motility rates have been reported. In contrast to the improvement in sperm motility, no effect on the VCL or LIN was noticed when using the PLGA system and GnRHa + Met.

The least effective of the tested treatments was the recombinant hCG at 20 μg kg^{-1} BW, approximately equal to 500 I.U., which produced results similar to the control group. A possible explanation could be the lower affinity of gonadotropin receptors for the mammalian recombinant protein, as has been suggested for other cyprinids [29,30].

The PLGA microparticles with continuous GnRHa release and GnRHa + Met both significantly increased blood plasma concentration of T and 11-KT compared to the control group. Androgens 11-KT and T are primary sex steroids responsible for the initiation and progression of spermatogenesis [31]. 11-KT stimulates the development of secondary sexual characteristics, spermatogonial proliferation, and spermiation [32], whereas T, a biosynthetic precursor of 11-KT, stimulates spermatogenesis [33]. Increased levels of 11-KT and T provide evidence of their roles in facilitating spermiation in bala sharks and underscore the positive effect of the PLGA microparticle system with continuous GnRHa release on the quantity and quality of bala shark sperm.

Release of GnRHa from PLGA microparticles occurs via diffusion and homogeneous bulk erosion of the biopolymer [13] and is characterized by an initial burst immediately after administration and a sustained or continuously declining release until depletion of the microspheres (Figure 1). We suggest that the high initial burst of GnRHa with subsequent decline might be the reason for the potent effect versus other treatments, as well as compared to the results of studies with other freshwater species that used systems with more gradual GnRHa release [17,18]. It is unclear whether constantly elevated plasma LH in treatments with GnRHa delivery systems reflects the natural physiologic situation necessary for gonadal steroidogenesis changes [5]; nevertheless, it seems to induce the appropriate hormonal changes for triggering gonad maturation and the production of viable sperm.

During preparatory work for this study, contamination of bala shark sperm by urine was observed, leading to premature sperm activation. To ensure the high fertilizing ability of Bala shark sperm, we strongly recommend the collection of sperm into immobilizing solution, e.g., Kurokura 180 [25], to counteract this problem.

5. Conclusions

Based on our data, we can conclude that a PLGA microparticle system (75% polylactic acid; 25% polyglycolic) with continuous release of 10 μg kg^{-1} of GnRHa in bala sharks is a potent inducer of sperm of both high quality and quantity and, together with the possibility of precise fish-specific dosing, represents an effective means of sperm induction. Another advantage of the PLGA delivery system with continuous GnRHa release in artificial reproduction of cyprinids might be the elimination of the so-far necessary addition of dopamine antagonists in cyprinid reproductive techniques. However, more research on sustained-release systems in freshwater fish reproductive techniques is needed.

Author Contributions: Conceptualization, P.P.; methodology, P.P., B.D., S.B., A.S., J.V., M.H.; software, S.B.; validation, P.P., B.D., J.V.; formal analysis, P.P.; investigation, P.P., J.K. (Jindřiška Knowles), J.K. (Jan Kouřil); resources, P.P.; data curation, B.D.; writing—original draft preparation, P.P., B.D.; writing—review and editing, B.D.; visualization, B.D.; supervision, P.P., B.D.; project administration, P.P.; funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Agriculture of the Czech Republic, grant number NAZV QK1810221.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and followed national and international guidelines for the protection of animal welfare (EU-harmonized Animal Welfare Act of the Czech Republic). The experimental unit is licensed with no. 2293/2015-MZE-17214 and no. 55187/2016-MZE-17214 within the project

NAZV QK1810221 according to the Czech National Directive (the Law against Animal Cruelty, no. 246/1992).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors give special thanks to Ing. Pavel Šablatura for his technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 8

General discussion

Hormonal stimulation of ovulation and spermiation is well established in aquaculture. A variety of exogenous hormones have been used in fish to address reproductive dysfunction or enhance reproductive output (Mylonas et al., 2017). The potential for sustained gonadotropin-releasing hormone analogue (GnRHa) release was recognized early in the development of intensive aquaculture (Fontenele, 1955), since final oocyte maturation and spermiation often require an extended period of hormone stimulation, administered in multiple injections (Slater et al., 1995). Re-injection is time consuming, labour intensive, and stressful to broodfish, potentially having a negative impact on reproductive output (Schreck, 2010), particularly in large fish or when they are kept outdoors in sea cages or ponds (Mylonas et al., 2007; Corriero et al., 2009). Various GnRHa delivery systems to control spawning have been tested in fish (Mañanós et al., 2008; Mylonas et al., 2010), but the use and impact of sustained GnRHa release in freshwater fish species has not been intensively studied (Mylonas and Zohar, 2000; Mylonas et al., 2010). The research reported in this thesis was carried out with the goal of developing a technology to ensure long-lasting release of GnRHa. Encapsulation of GnRHa increases its stability (Siepmann and Siepmann, 2006), counteracting the otherwise short lifetime in the blood stream (Gothilf and Zohar, 1991). Based on its biodegradability, wide availability, ease of adjustment of the drug and dosage properties, among other benefits (Vysloužil et al., 2016; Shi et al., 2020; Su et al., 2021;), we selected poly(lactic-co-glycolic acid) as a matrix to encapsulate GnRHa.

Poly(lactic-co-glycolic acid) microparticles were designed for intramuscular injection and created using $Water_1/Oil/Water_2$ technology, a common procedure for effective encapsulation of water soluble drugs (Zhang and Zhu, 2004; Jang et al., 2007). The microparticles exhibit a smooth surface, low porosity, and excellent sphericity. The most important property of microparticles is size. It is necessary to produce microparticles small enough to allow non-problematic and painless application (Park et al., 2019). Microparticles produced for our purposes had heterogeneous size distribution of tens of microns (less than 100 microns) allowing smooth injection (Yoo and Won, 2020). During the experimental application, the particles tended to show sedimentation and larger needles were used for intramuscular injection. Sedimentation of PLGA particles is typical when using a low viscosity solution such as water or saline solution (Sarmadi et al., 2020). To clarify the effect of mGnRHa encapsulated in PLGA microparticles, *in vivo* experiments were carried out in several fish species in specimens of both sexes.

Effect of PLGA microparticles on ovulation

Efficacy of PLGA microparticle-based treatment in females was evaluated in pikeperch *Sander lucioperca* and peled *Coregonus peled*. The selection of species was based on their high market value (Lappalainen et al., 2016; Stejskal et al., 2018; Sapozhnikova et al., 2020), use in intensive aquaculture, and the availability of a well-organized workforce.

Two types of PLGA microparticles created by Resomer RG 653H and Resomer RG753H were tested, producing similar effects on ovulation. A significantly higher ovulation rate was recorded in groups injected with PLGA microparticles (Chapters 3 and 4) compared to the control groups. High effectiveness of a low mGnRHa dose (5μg kg^{-1}) encapsulated in PLGA microparticles was observed in female pikeperch. This was unexpected, as high mGnRHa doses are commonly administered to stimulate ovulation in pikeperch (Křišťan et al., 2013; Żarski et al., 2013). The use of a lower dose of mGnRHa offers the potential for reduction in cost and minimizes risks of overdose (Rainis et al., 2003).

Efficacy of hormone treatment can be estimated by calculating rates of ovulation, fertilization, and fecundity (Fakriadis et al., 2020). Hormone treatment did not show enhancement in egg quality or quantity. The application of mGnRHa in PLGA microparticles to female fish resulted in adequate reproductive output in both tested species. High variation in egg quality was observed in pikeperch. This is a commonly reported problem in the artificial reproduction of pikeperch and remains a major bottleneck in its intensive aquaculture (Schaerlinger and Żarski, 2015; Żarski et al., 2019; Ljubobratović et al., 2022). Based on the outcome of our and other studies, it can be assumed that use of the PLGA microparticle had no direct negative effect on egg quality in pikeperch, and the problems are connected to thermal regime and physiological status of the females (Ljubobratović et al., 2022). No significant differences were found in the latency period or in the absolute and relative fecundity among hormone treatments in either peled or pikeperch. The reproductive performance values corresponded with findings of other authors (Żarski et al., 2012; Svinger and Kouril, 2014; Ljubobratović et al., 2022). Low influence of hormone stimulation on latency and fecundity were reported by other authors (Mikolajczyk et al., 2008; Falahatkar et al., 2013; Křišťan et al., 2013; Ljubobratović et al., 2022). Generally, hormone stimulation does not influence fecundity in synchronous spawning fish. However, in species with asynchronous ovarian development, fecundity can be increased by stimulating a greater number of ovulations during the spawning season (Mugnier et al., 2000; Guzmán et al., 2009; Fakriadis et al., 2019).

Steroid hormones are important indicators and modulators of various functions and play an important role in sex determination, stress response, neuroprotection, and reproduction (Guiguen et al., 2010; Rajakumar and Senthilkumaran, 2020). We used the enzyme linked immunosorbent assay (ELISA) to record steroid levels in blood following hormone administration. The ELISA method developed for our analysis purpose exhibited ideal attributes of precision, accuracy and specificity. The sensitivity of our technique corresponded to previously developed radioimmunoassay (Sangalang et al., 1978; Scott and Hylland, 2002).

The precursor to all steroids is cholesterol. Cholesterol can be transformed to pregnenolone, which is subsequently converted into steroids (Miller, 2013) including T, 11-KT, and E2 (Rajakumar and Senthilkumaran, 2020). 17β oestradiol regulates ovarian development, with its highest concentrations usually occurring during the oocyte growth period and its lowest immediately prior to ovulation (Nagahama and Yamashita, 2008). In our studies of female pikeperch and peled, the highest E2 concentrations were measured at the beginning of trials, with a decreasing trend towards ovulation. These results agree with reports in fish species such as common carp *Cyprinus carpio* (Levavi-Zermonsky and Yaron, 1986), tench *Tinca tinca* (Podhorec et al., 2016), and Persian sturgeon *Acipenser persicus* (Khara et al., 2014). The change corresponds to the anticipated natural shift in the biosynthetic pathway from the secretion of C19 to C21 steroids (Podhorec et al., 2016). In some cases, a slight elevation in plasma E2 concentration may occur due to high aromatase activity in the ovary upon GnRHa injection (Sharaf, 2012). Thus, the increase of E2 in the bloodstream after hormone administration can be associated with reduced initiation of ovulation (Mohammadzadeh et al., 2021).

Testosterone and 11-KT are the primary androgenic steroid hormones determined in males. In females, the role of 11-KT has not been fully clarified (Chaves-Pozo et al., 2008)). Testosterone is known to regulate the release and surge of luteinizing hormone and follicle stimulating hormone (Kumar et al., 2021; Slater et al., 1994) and is a common precursor of E2 (Lubzens et al., 2010). The high cost of analysis of 11-KT levels limited the procedure to female pikeperch in the present study. Levels of 11-KT were similar in all groups and increased towards ovulation, suggesting its importance in fish ovulation, but further investigation is needed to clarify any role in final oocyte maturation.

No abnormalities in hatched larvae were observed and no adverse effect of PLGA microparticles was discernible in any study, leading to the conclusion that treatment using PLGA does not pose a risk to broodstock or offspring.

Effect of PLGA microparticle treatment on spermiation

Generally, males receive less attention than females in study of fish reproduction (Fakriadis et al., 2020). In males, a major issue in aquaculture is diminished and unpredictable milt quantity and quality, which can negatively affect fertilization and larval development and should not be overlooked (Duncan et al., 2012; Mylonas et al., 2017). To facilitate sperm collection and increase its availability during the reproductive season, it is necessary to enhance spermiation and increase sperm quantity (Mylonas et al., 2017). Studies of male reproduction can improve broodstock management and develop suitable technology and methods to enhance spermiation (Mañanós et al., 2002).

In studies included in this thesis, viable spermatozoa were produced by all PLGA treatment groups with no significant differences among the hormone treated groups. Administration of PLGA microparticles was associated with a consistent increase of milt volume in pike and sterlet males. In Bala shark males, the milt production was significantly higher 24 hours post-injection, but no further sampling was conducted, as the sensitivity of this species could lead to high mortality (Lipscomb et al., 2018). In sterlet, hormone treatment with PLGA microparticles led to a prolongation of spawning, with release of sperm until the end of the trials. A prolonged spermiation period and elevated milt volume has been reported in species including Atlantic halibut *Hippoglossus hippoglossus* (Vermeirssen et al., 2004b), yellowtail flounder *Pleuronectes ferrugineus* (Clearwater and Crim, 1998), and Dace *Leuciscus leuciscus* (Cejko et al., 2012). It is known that controlled drug release can induce stimulation of milt production for up to few weeks. A single injection of GnRHa or CP increases milt volume and spermatozoon concentration for a few hours or days, followed by a dramatic decrease (Garcia, 1991; Rainis et al., 2003; Mylonas et al., 2017), while, for example in European seabass *Dicentrarchus labrax*, GnRHa implants and microsphere treatment results in significantly higher sperm volume for at least 35 days post-treatment (Sorbera et al., 1996). In other research, GnRHa implants stimulated spermiation for at least 27 days, while acute GnRHa treatment resulted in spermiation for only three days post-injection (Rainis et al., 2003). Sustained GnRHa release techniques, including with PLGA microparticles, have great potential, since they combine the properties of a highly resistant analogue with prolonged induction of spermiation, avoiding repeated injections and manipulation (Mylonas et al., 2017).

Spermatozoon concentration was increased in northern pike 48h post-injection in fish stimulated with CP and a combination of PLGA and dopamine agonist. Higher concentration of spermatozoa was observed in sterlet after PLGA microparticle hormone treatment throughout the experiment, while no effect on spermatozoon density was observed in Bala shark males. Enhanced spermatozoon density is a frequent result of hormone treatment because of greater hydration of the testes through increased seminal fluid production. The increased seminal fluid volume allows stripping of spermatozoa that previously could not be released. Therefore, more spermatozoa can be obtained over a longer period, and the concentration decreases significantly. However, in some species, spermatozoon density was reported to be increased after sustained GnRHa treatment. In this case, hormone treatment stimulated spermatogenesis, spermiogenesis, and spermiation (Schulz et al., 2010).

Hormone treatment often increases spermatozoon production in fish, while quality, i.e., velocity, motility duration, and percent motility, is usually not influenced (Mylonas et al., 2017). Spermatozoon quality was not negatively affected by the PLGA microparticle treatment. In

sterlet and Bala shark *Balantiocheilos melanopterus*, no significant differences were found among acute and PLGA hormone-treated group, while, in northern pike, significant increases in average path velocity and curvilinear velocity were observed 96 hours post-injection in all hormone treated groups. In Atlantic halibut, sustained GnRHa release increased sperm volume with a concomitant decrease in spermatozoon density and prolonged increase in motility percentage (Vermeirssen et al., 2004a). Our results confirmed that sustained GnRHa release can improve milt production without adverse impacts on the quality and fertilizing ability of the sperm (Mylonas et al., 1997a).

Spermatozoon motility rate is a valuable marker of quality, as spermatozoa must actively swim to reach the micropyle of the egg. Motility rate was significantly greater in sterlet treated with a low dose of mGnRHa in PLGA microparticles compared to the group receiving a higher dose. A negative influence of high doses on final maturation and gamete quality has been confirmed in other teleosts (Fernandez-Palacios et al., 2014; Podhorec et al., 2016). In northern pike, motility increased in PLGA groups compared to initial values, while, in Bala shark, a significant increase in spermatozoon motility was observed in those treated with combination of PLGA and metoclopramide. A positive influence of sustained GnRHa release on motility had been observed in yellowtail flounder *Pleuronectes ferrugineus* (Clearwater and Crim, 1998) and Atlantic halibut (Vermeirssen et al., 2000). Our results indicate that, although milt volume and spermatozoon production may be positively affected by sustained GnRHa release, the motility characteristics of the spermatozoa remain unchanged.

The PLGA treatment was shown to increase and maintain androgen concentrations. Androgen 11-KT is the major regulator of spermatogenesis and spermiogenesis (Miura and Miura, 2003). In some teleosts, for example striped bass *Morone saxatilis* (Mylonas et al., 1997b) and greenback flounder *Rhombosolea tapirina* (Lim et al., 2004), increased plasma levels of T and 11-KT with sustained GnRHa release correlated with higher milt volume. Nevertheless, the increased androgen levels have not been linked to elevated milt volume in all fish species (Mylonas et al., 1997a; Clearwater and Crim, 1998; Mañanós et al., 2002). Thus, the androgen levels may not be a factor in the GnRHa-induced higher milt volume (Fakriadis et al., 2020). 17β oestradiol is the major steroid in female fish but is also present in males. Plasma E2 concentration did not differ in male northern pike, indicating that this steroid does not play a significant role in the process of spermiation.

In general, hormonal stimulation for enhancement of spermiation may positively influence spermatozoon quality in species with viscous sperm or that do not produce releasable sperm. However, in the majority of fish, hormone therapy has no effect on sperm quality (Mylonas et al., 2017).

The study comprising this thesis demonstrated that spermiation can be enhanced with the injection of mGnRHa carried in PLGA microparticles in some fish species. The hormone administered in microparticles stimulated sperm production without adversely affecting sperm parameters. Milt of enhanced quantity and standard quality can be produced over an extended period, effectively prolonging the spawning season in cultured fish.

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English summary

Using of prolonged releasing drug systems in fish reproduction

A key factor in sustainable aquaculture is consistent production of high-quality sex products followed by successful spawning and rearing of larvae. The application of hormone preparations is used to stimulate the final maturation of gametes and to optimize artificial reproduction. There is currently a need to develop techniques to overcome reproductive dysfunction and resolve complications of artificial reproduction. Controlled drug release systems have the potential to overcome challenges in the culture of economically important fish species.

The aim of the research encompassed in this dissertation was to develop and validate an innovative method of hormone stimulation using PLGA microparticles for sustained release of a synthetic analogue of mammalian gonadotropin-releasing hormone in several economically important fish species. Since this is the first record of the use of PLGA microparticles in fish, the focus was to establish the optimal dose of the active substance, quantify the steroid response of the broodstock, and to assess the quality and quantity of obtained sex products.

To evaluate the effect of PLGA microparticles, those fish species commonly reared in intensive and pond aquaculture were selected for study. Fish of both sexes were included to determine the effect of the treatment on the production and quality of eggs as well as on the quality and quantity of sperm.

The use of mGnRHa encapsulated in PLGA microparticles showed no negative effects on the quality of the obtained sex products or larvae. An interesting finding was the high efficacy of a low dose of mGnRHa encapsulated in microparticles in inducing ovulation in pikeperch females, in which a higher dose of GnRHa has traditionally been standard.

The results of this research provide valuable information about the formation, use, and outcomes of PLGA microparticles as a system for long-term release of GnRHa. The method is suitable for commercially important fish species and carries a number of advantages over acute hormone stimulation, including eliminating the necessity of repeated injections and reduction in the total effective GnRHa dose compared to other hormone preparations, meaning less stress to fish and lower costs to fish producers. This knowledge is of value both for further research in the field of sustained drug release and for practical use in intensive and pond aquaculture.

Czech summary

Využívání dlouhodobého uvolňování léčiv v reprodukci ryb

Jedním z klíčových faktorů pro prosperující a udržitelnou akvakulturu je zajištění stabilní produkce kvalitních pohlavních produktů, následný úspěšný umělý výtěr a odchov larev. Aplikace různých hormonálních preparátů je velmi často využívána ke stimulaci finálního zrání gamet a optimalizaci umělé reprodukce. V současné době je potřeba vyvinout techniky k překonání reprodukčních dysfunkcí a řešení komplikací spojených s umělou reprodukcí. Systémy pro kontrolované uvolňování léčiv mají potenciál překonat překážky v rozmnožování hospodářsky významných druhů ryb.

Cílem této dizertační práce bylo vyvinout a ověřit inovativní metodu hormonální stimulace za využití PLGA mikročástic jako systému pro protrahované uvolňování syntetického analogu savčího gonadotropin uvolňujícího hormonu u několika ekonomicky významných druhů ryb. Vzhledem k tomu, že se jedná o první záznam použití PLGA mikročástic u ryb, důraz byl kladen zejména na studium optimální dávky účinné látky, steroidní odezvu generačních ryb a kvalitu a kvantitu získaných pohlavních produktů. K hodnocení účinku PLGA mikročástic byly zvoleny takové druhy ryb, které reprezentují skupiny ryb běžně chované v intenzivní a rybniční akvakultuře. Ke studiu byly vybrány ryby obojího pohlaví, což následně umožnilo určit vliv tohoto ošetření jak na produkci a kvalitu jiker, tak i na kvalitu a kvantitu získaného spermatu od mlíčáků.

Z výsledků dizertační práce vyplývá, že hormonální stimulace mGnRHa enkapsulovaného do PLGA mikročástic neměla žádný negativní vliv na kvalitu získaných pohlavních produktů a larev. Zajímavým zjištěním byla vysoká účinnost nízké dávky mGnRHa enkapsulovaného v mikročásticích na indukci ovulace u jikernaček candáta obecného, kde jsou preferovány z pravidla vyšší dávky samostatného GnRHa.

Je možné konstatovat, že výsledky této dizertační práce poskytují cenné informace o formaci, použití a vlivu PLGA mikročástic jako systému pro dlouhodobé uvolňování GnRHa. Tyto znalosti mají vysoký přínos jak pro další výzkum v oblasti protrahovaného uvolňování léčiv, tak i pro praktické využití v intenzivní a rybniční akvakultuře, neboť bylo prokázáno, že využití této hormonální stimulace je vhodné u mnoha komerčně významných druhů ryb a nese sebou řadu výhod ve srovnání s akutní hormonální stimulací. Mezi tyto benefity pak patří například eliminace použití opakovaných injekcí, čímž dochází ke zlepšení welfare chovaných ryb a snížení celkové účinné dávky GnRHa ve srovnání s jinými hormonálními preparáty. Pořízení GnRHa je zpravidla velmi ekonomicky nákladné a redukce jeho celkové účinné dávky by v případě budoucí masové produkce PLGA mikročástic znamenalo i pokles finančních nákladů vynaložených na hormonální stimulaci ovulace a spermiace.

Acknowledgements

It is my great pleasure to express my warm thanks to M.Sc. Peter Podhorec, Ph.D., for being such a great supervisor and providing friendly guidance through the years, always willing to help, discuss, and develop ideas. I greatly appreciate the level of freedom during my study, while being always supported and positively motivated by Peter.

I would like to thank all the staff of the Laboratory of Controlled Fish Reproduction and Intensive Fish Culture and other laboratories of the Faculty of Fisheries and Protection of Waters in Vodňany, who provided assistance with experiments and lab work. Particular thanks go to Vlastimil Stejskal, Jan Kouřil, and Anna Ivanova who gave tremendous help throughout the years.

Nobody has been more important to me in the pursuit of this goal than the members of my family. I would like to thank my parents, Dagmar and Jindřich, whose love and guidance are with me in whatever I pursue (Mami a tati, bez Vás by to nešlo. Díky!). Most importantly, I wish to thank my loving and supportive husband, Nikolai, and my two wonderful children, who provide unending inspiration as well as happy distractions to rest my mind outside of my research – **to you I dedicate this thesis.**

I also appreciate the financial support from the following projects that funded parts of the research discussed in this dissertation:

- Project no. CZ.1.05/2.1.00/01.0024 CENAKVA – South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (project leader prof. Otomar Linhart)
- Project no. LO1205 under the NPU1 program CENAKVA II - South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (project leader prof. Otomar Linhart)
- Project no. CZ.0.2.1.01/0.0/15_003/0000495 – The Ministery of Education, Youth and Sports of the Czech Republic (project leader Jaroslav Turánek, Ph.D.)
- Project NAZV no. QK1810221, Ministry of Agriculture of the Czech Republic (project leader Peter Podhorec, Ph.D.)
- Project NAZV no. QK1920326, Ministry of Agriculture of the Czech Republic (project leader Peter Podhorec, Ph.D.)
- GAJU 099/2019/Z Effect of PLGA microparticles on induction of ovulation in pikeperch (project leader Dipl.-Ing. Jindřiška Knowles)
- GAJU 060/2016/Z Inovace pro dlouhodobě udržitelný rozvoj akvakultury (project leader Assoc. Prof. Jan Mráz)

List of publications

Peer-reviewed journals with impact factor

Herrera, F., Boryshpolets, S., Mraz, J., **Knowles, J.**, Bondarenko, O., 2022. Pikeperch (*Sander lucioperca*) spermatozoa motility and volume regulation under different osmotic and ionic conditions. Fish Physiology and Biochemistry 48, 899–910. (IF 2021 = 3.014, AIS 2021 = 0.482)

Knowles, J., Boryshpolets, S., Kholodnyy, V., Rahi, D., Vysloužil, J., Muselík, J., Stejskal, V., Kouřil, J., Podhorec, P., 2022. Effects of gonadotropin-releasing hormone agonist administered in microparticles on sperm quality and quantity, and plasma sex steroid levels in northern pike. Animal 16, 100430. (IF 2021 = 3.730, AIS 2021 = 0.643)

Knowles, J., Vysloužil, J., Policar, T., Milla, S., Holická, M., Podhorec, P., 2022. Spawning performance and sex steroid levels in female pikeperch *Sander lucioperca* treated with poly(lactic-co-glycolic acid) microparticles. Animals 12, 208. (IF 2021 = 3.730, AIS 2021 = 0.643)

Podhorec, P., **Knowles, J.**, Vysloužil, J., Boryshpolets, S., Sotnikov, A., Holická, M., Kouřil, J., Dzyuba, B., 2022. The effect of hormonal treatment on selected sperm quality parameters and sex steroids in tropical cyprinid bala shark *Balantiocheilos melanopterus*. *Fishes 7*, 122. (IF 2021 = 3.170, AIS 2021 = 0.503)

Holická, M., Vysloužil, J., Kubová, K., Muselík, J., Radinova, E., Vetchý, D., Kotolová, H., Hammer, T., Mašek, J., Podhorec, P., **Knowles, J.**, 2021. Pre-formulation design of sustained-release GnRHa-loaded PLGA microspheres and associated formulations for controlling reproduction in aquaculture. Acta Poloniae Pharmaceutica 78, 801–812. (IF 2020 = 0.330, AIS 2020 = 0.088)

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Matejkova, J., Podhorec, P., 2019. Sustained drug delivery system in fish and the potential for use of PLGA microparticles: a review. Veterinarni Medicina 64, 287–293. (IF 2018 = 0.636, AIS 2018 = 191)

Abstracts and conference proceedings

Knowles, J., Podhorec, P., 2019. Effect of PLGA microparticles and carp pituitary on northern pike *Esox lucius* spermiation stimulation and its effect on quality and quantity of sperm. 7–10 October 2019, Berlin, Germany. (Poster presentation)

Knowles, J., Podhorec, P., 2019. Effect of PLGA microparticles on reproduction of pikeperch *Sander lucioperca* and changes in the plasma level of reproductive hormones. 7–10 October 2019, Berlin, Germany. (Poster presentation)

Application of methods, patents, verified techniques

Knowles, J., Podhorec, P., Vysloužil, J., 2021. Usage of preparations based on microparticles loaded with hormonal substances in aquaculture. FFPW USB, Vodňany, Edition of Methodologies, no. 191, 44 pp. (in Czech)

Podhorec, P., **Knowles, J.**, Vysloužil, J., Boryshpolets, B., Rodina, M., Dzyuba, B., 2021. Hormonal stimulation of spermiation in rheophilic fish species of small sturgeon and balla shark using PLGA microparticulate systems with controlled release of gonadoliberin. FFPW USB, Vodňany, Edition of Methodologies, no. 194, 26 pp. (in Czech)

Knowles, J., Podhorec, P., 2020. Induction of ovulation in pikeperch (*Sander lucioperca*) by using preparations based on PLGA microparticles loaded with mGnRHa. FFPW USB, Vodňany, Edition of Methodologies no. 175, 30 pp. (in Czech)

Training and supervision plan of study

Name	Jindřiška Knowles
Research department	2016–2022 – Laboratory of Controlled Fish Reproduction and Intensive Fish Culture (IAPW of FFPW)
Supervisor	Peter Podhorec, Ph.D.
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Ph.D. courses	**Year**
Biostatistics	2017
Intensive fish culture	2017
Fish reproduction	2017
Ichthyology and fish taxonomy	2019
English language	2018

Scientific seminars	**Year**
Seminar days of IAPW and FFPW	2017 2018 2019 2021

International conferences	**Year**
Knowles, J., Podhorec, P., 2019. Effect of PLGA microparticles on reproduction of pikeperch *Sander lucioperca* and changes in the plasma level of reproductive hormones. Aquaculture Europe 2019, 7–10 October 2019, Berlin, Germany	2019
Knowles, J., Podhorec, P., 2019. Effect of PLGA microparticles and pituitary extract on northern pike *Esox lucius* spermiation and its effect on quality and quantity of sperm. Aquaculture Europe 2019, 7–10 October 2019, Berlin, Germany	2019

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Sylvain Milla, Ph.D., University of Lorraine, Animal and Functionality of Animal Products, enzyme-linked immunosorbent assay of sex steroid hormones, one week	2018
Dr. Nicole Rhody, Mote Marine Laboratory & Aquarium, Florida, USA, marine recirculating systems, artificial reproduction of common snook and longfin yellowtail, two months	2018

Pedagogical activities	**Year**
• Introduction at summer school of the project entitled Effect of long-acting PLGA microparticles on plasma level of steroids in northern pike *Esox lucius*	2019
• Lecture to bachelor and masters students in "Aquaristics," ~90 hours	2017–2022
• Consultant to Oldřich Pecha for Bachelor's thesis entitled "The use of preparations for the controlled release of hormones in the artificial reproduction of fish" defended at FFPW USB	2017–2019

Curriculum vitae

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2016 – Present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2013–2016	Dipl.-Ing., Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2010–2013	B.Sc., Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2006–2010	Střední rybářská škola, Vodňany, Czech Republic

COMPLETED COURSES

Biostatistics, Ichthyology and fish taxonomy, English language, Fish reproduction, Intensive fish culture

RESEARCH STAYS AND COLLABORATIONS

09/2014 – 11/2014	Akva-Tek su ürünleri, Turkey, Dr. Nedim Yazicioglu
19/8 – 25/8/2018	University of Lorraine, Animal and Functionality of Animal Products, Sylvain Milla, Ph.D.
09/2019 – 11/2019	Mote Marine Laboratory & Aquarium, USA, Florida, Dr. Nicole Rhody