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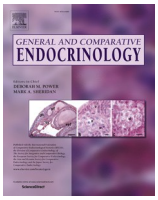

Biocompatible polymeric microparticles serve as novel and reliable vehicles for exogenous hormone manipulations in passerines

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ABSTRACT

The administration of exogenous hormones emerged as an essential tool for field studies in endocrinology. However, working with wild animals remains challenging, because under field conditions not every available method meets the necessary requirements. Achieving a sustained elevation in hormone levels, while simultaneously minimising handling time and invasiveness of the procedure is a difficult task in field endocrinology.

Facing this challenge, we have investigated the suitability of biocompatible polymeric microparticles, a novel method for drug-administration, as a tool to manipulate hormones in small songbirds. We chose the insulin-like growth factor-1 (IGF-1) as target hormone, because it receives great interest from the research community due to its important role in shaping life-history traits. Moreover, its short half-life and hydrophilic properties imply a major challenge in finding a suitable method to achieve a sustained, systemic long-term release. To study the release kinetics, we injected either IGF-1 loaded polylactic-*co*-glycolic acid (PLGA) microparticles or dispersion medium (control group) in the skin pocket of the interscapular region of captive bearded reedlings (*Panurus biarmicus*). We collected blood samples for 7 consecutive days plus an additional sampling period after two weeks and complemented these with an *in vitro* experiment.

Our results show that *in vitro*, PLGA microparticles allowed a stable IGF-1 release for more than 15 days, following a burst release at the beginning of the measurement. *In vivo,* the initial burst was followed by a drop to still elevated levels in circulating IGF-1 until the effect vanished by 16 days post-treatment.

This study is the first to describe the use of PLGA-microparticles as a novel tool for exogenous hormone administration in a small passerine. We suggest that this method is highly suitable to achieve the systemic long-term release of hydrophilic hormones with short half-life and reduces overall handling time, as it requires only one subcutaneous injection.

1. Introduction

Exogenous hormone administration is a frequently used method to experimentally test for the ecological relevance of variations in hormone levels in different organisms (Bonier and Cox, 2020; Crim, 1985; Fusani, 2008; Sopinka et al., 2015). Techniques that have previously been developed and used in laboratories (e.g., in pharmacology) recently became affordable and accessible to be safely applied to a variety of animal species in natural environments. The administration of hormones in a controlled setting (e.g., laboratory or housing facilities) allows standardised conditions (i.e., temperature, dietary regime, photoperiod), regular access to the target individuals, frequent handling, and strict monitoring of the treatment effects. In contrast, in wild, free-ranging animals, experiments involving manipulations of hormone levels face several pitfalls and limitations (Fusani, 2008; Fusani et al., 2005; Ovid et al., 2018; Quispe et al., 2015).

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There are several techniques for the administration of hormones, which differ greatly in their practicality for field studies. The requirements are mainly determined by the invasiveness of the application process (e.g. handling time, surgery,..) and the efficacy and duration of the hormone release (reaching from hours to several weeks) (Fusani, 2008; Müller et al., 2009; Ovid et al., 2018; Quispe et al., 2015; Vitousek et al., 2018). Oral and topical administration are minimally invasive, but like injections (intramuscular, subcutaneous or intraperitoneal), result in a transient elevation of hormone levels as many commonly applied hormones, including steroid hormones such as corticosterone and testosterone, have a short half-life (Birrenkott and Wiggins, 1984; Goymann et al., 2002; Lõhmus et al., 2006; Vitousek et al., 2018). The sustained elevation requires daily, controlled application, which is not feasible in many field studies (Ovid et al., 2018; Sopinka et al., 2015). The repeated capture of target individuals or controlled supplemental feeding is often not possible or would interfere with the natural behaviour of the animals (e.g., induce desertion, emigration from the site, interruption of mating or feeding behaviour) (Colwell et al., 1988; Criscuolo, 2001; Fusani et al., 2005; Lõhmus et al., 2006; Ovid et al., 2018; Putman, 1995). In contrast, subcutaneous or intraperitoneal implants facilitate a long-term release of the target hormone, but require at least one surgical procedure, which increases handling disturbance. Prolonged handling affects the physiological stress response, which is also tightly linked to the condition and life-history stage of an individual, both factors that are difficult to control for in field conditions (Colwell et al., 1988; Criscuolo, 2001; Crossin et al., 2016; Huber et al., 2021; Lattin et al., 2012; Ovid et al., 2018; Putman, 1995). Wild, free-ranging animals also have less capacity to recover from prolonged or repeated capture events as they have limited access to food, face competition over resources and encounter predators. Consequently, animals in natural conditions are more likely to suffer a potential decrease in fitness and/or survival than in captivity (Bonier and Cox, 2020; Fusani et al., 2005; Goutte et al., 2010; Huber et al., 2021; Lattin et al., 2012; Putman, 1995; Spée et al., 2011). Finally, and contrary to many experiments in a clinical or preclinical setting, studies focusing on an ecological context of a given physiological response often aim to mimic hormone levels that correspond to a specific physiological phenotype within a population. The long-term release of the hormone into the bloodstream and/or tissue is often desirable to achieve hormone levels within the population's estimated natural range (Beck et al., 2016; Fusani, 2008; Ovid et al., 2018; Quispe et al., 2015).

As Fusani (2008) reviewed, many pioneering studies on field endocrinology have been conducted on passerine species. Birds, in particular songbirds, have emerged as a popular study system because they are found throughout most of the world and express a large diversity of life-history traits. Many avian species appear as easy to observe, because they display conspicuous ornamental traits (e.g., plumage ornaments) and behaviours that have been measured and categorized over the past centuries. In addition, many of the common model species (e.g., great tits (*Parus major*)) seem to cope well with handling and blood sampling, despite their often small size (Gaunt et al., 1997; Huber et al., 2021; Owen, 2011; Voss et al., 2010).

There are now a variety of safe options available to steadily release exogenous hormones over a specified timeframe (Beck et al., 2016) and they have been tested sufficiently on passerine species in the field and the laboratory (Fusani, 2008; Müller et al., 2009; Ovid et al., 2018; Quispe et al., 2015; Vitousek et al., 2018). The most common method is the intraperitoneal or subcutaneous implantation of sterile, hormone-packed silastic tubes, which serve as reliable vehicles for hormone manipulations and cause long-lasting changes in peripheral hormone levels over several weeks. There are three frequently discussed downsides that include: i) an initial spike after implantation causing an elevation of the target hormone, sometimes far above natural levels, ii) silastic tubes are recommended to be removed after some time and therefore require repeated capture and handling, and iii) there are potential implications for animal welfare (i.e., capture stress or repeated wounding from surgery) (Fusani, 2008; Horton et al., 2007; Müller et al., 2009; Quispe et al., 2015). An alternative is the use of biodegradable controlled-release pellets designed to provide a steady release of hormones in small mammals. However, in avian species their release kinetics are characterized by a pronounced initial peak and a rapid depletion of the exogenous hormone, possibly due to the high avian body temperature and metabolism (Müller et al., 2009; Vágási et al., 2018). Another drawback is that they are only commercially available and are relatively expensive, but more cost-efficient alternatives have been developed recently, using in-house made pellets from beeswax or hardened peanut oil (Beck et al., 2016; Quispe et al., 2015). Yet many important peptide hormones are water-soluble and cannot be easily incorporated into the lipid matrix of the beeswax/peanut oil pellets. Another technique that can achieve a sustained release of hormones is osmotic mini-pumps, which are implanted into the animal and function based on an osmotic pressure difference between an osmotic layer within the pump and the surrounding tissue (Sinha and Trehan, 2003). While these devices may control the release of theoretically any substance, due to their relatively large size and expensive price, they did not become widespread in field endocrinology (Pedersen and Saether, 1999). An additional, yet important limitation of all pellets, tubes, and mini-pumps is that their initial implantation requires surgery (mini-pumps and silastic tubes even require additional surgery for removal), and the relatively rigid material and shape might constitute an irritation for the animal. Although all the hormone administration techniques discussed above, are considered safe and reliable, the frequent use of hormone manipulations in the field, together with an increasing awareness of animal welfare require the improvement and development of new methods that are less invasive while allowing an effective long-term elevation of target hormones.

Here we test a novel, minimally invasive technique for exogenous hormone administration. We use a single subcutaneous injection of polylactic-*co*-glycolic acid (PLGA) microparticles to achieve a steady hormone release over several days. PLGA is a versatile co-polymer of lactic and glycolic acid that is clinically used as resorbable suture material and technologically for preparing drug delivery systems such as nano- or microparticles (Wischke and Schwendeman, 2008). The use of PLGA for the protection and controlled delivery of proteins is well-established (Cohen et al., 1991; Dördelmann et al., 2014; Kim et al., 2006). It is a biodegradable and biocompatible polyester that degrades to non-toxic lactic acid and glycolic acid to finally yield carbon dioxide and water (Makadia and Siegel, 2011). The degradation profile can be modified by the molecular weight of the polymer and the ratio of glycolic and lactic acid in the molecule (Park, 1995). By entrapping drugs within a PLGA matrix, a continuous and controlled release profile can be achieved. Ideally, this reduces the frequency of drug administration and ensures a constant plasma level (Stevanović and Uskoković, 2009). Furthermore, a biodegradable matrix material such as PLGA avoids the need for surgical removal of depleted delivery systems (Sinha and Trehan, 2003).

We used insulin-like growth factor 1 (IGF-1) as the target substance. It is of increasing interest to many fields of the life sciences because it is an evolutionarily highly conserved metabolic hormone. It regulates a large array of physiological processes and has been shown to be associated with the development of major life-history traits (Al-Samerria and Radovick, 2021; Dantzer and Swanson, 2012; Lodjak and Verhulst, 2020). One crucial aspect in working with IGF-1 is its short half-life in the circulation (in chicken, ~ 32 min, regardless of the dose), which requires a suitable delivery system/vehicle to achieve a steady release and hence elevation of IGF-1 *in vivo* (Hameed et al., 2019; Sun et al., 2019; Zhang et al., 2020). While a growing number of preclinical and clinical studies addressed this issue by using PLGA microparticles to increase systemic or local (i.e., specific target tissue) IGF-1 levels in laboratory or farm animals (Hameed et al., 2019; Haney et al., 2018; Lam et al., 2000; Meinel et al., 2001; Yuksel et al., 2000; Zhang et al., 2020), this method has never been validated in a wild animal, let alone

in a passerine species. To the best of our knowledge, only one experiment in captivity brought strong indication for the suitability of PLGA microparticles as a minimally invasive tool to increase IGF-1 in the bearded reedling (*Panurus biarmicus*), a common, small (~15 g) Eurasian songbird (Lendvai et al., 2021).

There is only a handful of studies examining the role of IGF-1 in regulating life-history traits of wild animal species, and even fewer testing the effects of an experimental increase of systemic IGF-1 on their physiology and behaviour (Dantzer and Swanson, 2012; Lodjak and Verhulst, 2020; Montoya et al., 2022). In the bearded reedling, IGF-1 has been studied in various contexts and is associated with regulating the stress response (Tóth et al., 2018), ornament expression (Mahr et al., 2020) and feather moult (Lendvai et al., 2021). Lendvai et al. (2021) has shown that one single subcutaneous injection of PLGA microparticles loaded with IGF-1 resulted in a significant elevation of IGF-1 on day 1 post-injection and was sufficient to alter moult patterns and feather quality. Although the findings by Lendvai et al 2021 confirm the physiological effect of the IGF-1 administration, there is no detailed information on the *in vivo* and *in vitro* release kinetics available as only two blood samples (after 24 h and 4 days) were collected after the treatment.

We now aim to investigate the release kinetics of PLGA microparticles loaded with IGF-1 in more detail, using captive bearded reedlings as models. The *in vivo* data will further be complemented with an *in vitro* study. The information we gain through this approach might provide novel and important insights into a promising method that increases hormone levels over the long term, while simultaneously avoiding supra-physiological hormone concentrations and decreasing handling time and disturbance.

2. Methods

All procedures were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna in accordance with the Universitýs guidelines for Good Scientific Practice and authorized by the Austrian Federal Ministry of Education, Science and Research (§26 des Tierversuchsgesetzes 2012-TG 2012: GZ 2020-0.292.788, GZ 2020-0.466.191 and A4/NN.AB-10087-22-2020) in accordance with current legislation.

2.1. General methods and housing conditions

All birds were juveniles and originated from a large population of wild, free-living bearded reedlings (3 study sites in the Lake Neusiedl region, Burgenland, Austria: Winden am See 47°55′57.2″N 16°46′26.4″E; Jois 47°56′32.9″N 16°47′32.1″E; Breitenbrunn 47°55′25.4″N 16°45′29.9″E). They were caught using mist-nets and playback equipment. Immediately after the capture, a small blood sample was drawn. Since handling stress might affect circulating IGF-1 levels, the exact capture time (i.e., the bird hitting the net) was noted, and blood samples were drawn within 3 min. Subsequently, the birds were transferred to cloth cages (each cage was provided with water, mealworms and apples) and transported to the outdoor housing facilities of the KLIVV (Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria).

They were housed in large outdoor aviaries, furnished with reed-bundles mimicking their natural environment. Food (fresh mealworms, insectivorous food containing quark, apples, carrots, dried insects, egg-food) and water were provided *ad libitum*. In addition, the birds received a piece of apple, millet and vitamin supplements (Korvimin®ZVT + Reptil from WDT eG, Garbsen, Germany) every week. Three days before the start of the experiment, birds were transferred into cages (100 × 50 × 50 cm) located in indoor housing facilities. This period is considered to be a sufficient acclimatisation time, because previous experience with this species has shown, that bearded reedlings quickly adapt to captive conditions (Lendvai et al., 2021; Mahr et al., 2020). In addition, daily observations indicated that all birds resumed feeding, preening and social interactions within a short period after the transfer into the experimental cages (own observations). In this species, separate housing is not recommended, because reedlings are social animals and separation may lead to stress (Griggio and Hoi, 2011; Tóth et al., 2018). Therefore, each experimental cage contained 5 birds that were randomly assigned to the cages and experimental rooms. Although the juveniles were not displaying aggressive interactions, each cage was provided with 3 feeders and 2 water dispensers to guarantee sufficient access to food and water. The indoor facilities were equipped with a ventilation system that allowed natural airflow, with temperatures resembling ambient climatic conditions. Similarly, light conditions were adjusted to the current conditions in the natural environment (light between 0600 and 1930).

2.2. IGF-1 microspheres preparation

Poly-(lactide-*co*-glycolide) RG502H (PLGA) was obtained from Evonik Nutrition & Care GmbH (Essen, Germany). Human recombinant insulin-like growth factor 1 (IGF-1) was bought from PreproTech EC, ltd. (London, UK). Polyvinylalcohol (PVA, 87–90 % hydrolysed, 30–70 kDa), dichloromethane (DCM) and all further chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich (St. Louis, MO, USA).

Microparticles were prepared by a double emulsion solvent-evaporation technique with modifications as initially described by Meinel et al. (2001). This technique is especially useful when the substance that needs to be incorporated is highly water soluble and is therefore mostly employed when preparing protein or peptide loaded PLGA particles (Cruz et al., 2021; Dördelmann et al., 2014; Lagreca et al., 2020). The method involves the preparation of two consecutive emulsions. The first one being a water-in-oil (w1/o1) emulsion where the aqueous core contains the protein or peptide drug together with stabilizing agents and the outer, organic phase contains the dissolved polymer. In the next step this first w1/o1 emulsion is then transferred into an aqueous phase of larger volume to yield the final double emulsion (w1/o1/w2). During this step, the polymer precipitates on the surface of the aqueous droplets of the first aqueous inner phase. The removal of the organic solvent then solidifies the polymer particle structure and yields the final particle suspension of PLGA particles entrapping a hydrophilic substance. In this study, the inner aqueous phase (w1) consisted of 20 µg IGF-1, 10 mM sodium-succinate and 140 mM NaCl dissolved in 150 µl purified water. Additionally, 2.5 mg bovine serum albumin and 2.0 mg succinylated gelatine (Gelofusin®) were added to stabilize IGF-1 during particle preparation and to control release. The efficacy of those compounds was previously investigated by Meinel et al. (2001). The aqueous phase w1 was then injected into a solution of 50 mg PLGA RG502H dissolved in 2 ml DCM and emulsified via ultrasonication using a Bandelin HD70 Sonoplus sonifier for 15 s at 30 % amplitude to yield emulsion 1. Subsequently, emulsion 1 was added to 30 ml 5 % aqueous PVA solution (w/v) and stirred for one minute at 500 rpm yielding the final w1/o/w2 double emulsion. For particle hardening, the final dispersion was diluted with 400 ml of purified water and stirred for 30 min at 100 rpm and under constant airflow to ensure complete evaporation of DCM. The microparticles were washed three times by centrifugation at 1068×g for 10 min and resuspension of the particle pellet in purified water. The purified microparticles were freeze-dried for at least 24 h and stored at 4 °C until further use. To assure low microbial contamination, all glassware used for particle preparation was autoclaved and the aqueous solutions were sterilized by filtration prior to use. Further, whenever possible, working steps were carried out in laminar air flow.

Prior to animal application, IGF-1 loaded microparticles were suspended in a dispersion medium yielding a final IGF-1 concentration of 280 ng/100 µl. The dispersion medium consisted of 1.5 % (w/w) carboxymethylcellulose, 5 % (w/w) mannitol and 0.02 % (w/w) polysorbate 80 dissolved in a sterile, physiologic sodium chloride solution.

2.3. Characterisation of IGF-1 containing PLGA-microparticles and quantification of IGF-1 by HPLC analysis

The particle size distribution of the microparticle suspension was determined by laser diffraction using a Mastersizer 3000 with a Hydro SV dispersion unit (Malvern Instruments, Malvern, UK). Lyophilised microparticles were suspended in purified water prior to particle size characterisation. The suspension was stirred at 700 rpm during the measurement. Further, particle size and morphology were analysed via light microscopy using a Zeiss Epifluorescence Axio Observer.Z1 deconvolution microscopy system.

Scanning electron microscopy was performed to examine the morphology of PLGA microparticles. The lyophilised microparticles were placed on aluminium SEM stub and high vacuum secondary electron imaging was performed using an Apreo vS SEM (Thermo Scientific, The Netherlands) at 1.0 kV.

IGF-1 was quantified using a Shimadzu Nexera XR HPLC (Shimadzu, Kyoto, Japan) equipped with an analytical CN-RP column (NUCLEODUR 100–5, CN-RP, 150 × 4.6, 5 μm column; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and a UV-diode-array detector. A gradient elution protocol with acetonitrile (ACN) and purified water at a 0.8 ml/min flow rate was employed. Formic acid was added to both solvents at a concentration of 0.1 %. Over ten minutes, the eluent was changed from 5 % ACN and 95 % water to 45 % ACN and 55 % water. IGF-1 was detected at 214 nm and the amount was calculated from a standard curve. To quantify encapsulated IGF-1, a certain amount of particles was dissolved in 1 ml of DCM + acetone (3 + 7). The sample was then centrifuged at 20817 × g for five minutes and the supernatant was discarded. This purification procedure was repeated three times. The final residue was dissolved in 0.1 M acetic acid and analysed by HPLC as described above.

For in-vitro IGF-1 release experiments, a dispersion of 40.0 mg microparticles in one ml PBS with Ca^{2+}/Mg^{2+} pH 7.4 were incubated under constant agitation at 37 °C. The body temperature of birds is several degrees (~41 °C) higher than the chosen incubation temperature of the *in vitro* experiments suggesting a faster liberation *in vivo*. However, IGF-1 is highly soluble in aqueous surrounding and accordingly the dissolution behaviour is not strongly affected by a slight change in temperature during the experiment. Furthermore, the viscosity of the dispersion medium and the coalescence behavior of microparticles that occur to some extent under *in vivo* conditions slow down the IGF-1 release. Hence, the estimated accelerating effect of the higher temperature *in vivo* on the release kinetics is weakened and IGF-1 liberation resembles more the release kinetics observed *in vitro* at lower temperature. The IGF-1 concentration in the acceptor medium was determined in regular intervals. At each time point, 200 μl of the acceptor medium were withdrawn and replaced by fresh PBS buffer. The aliquot was lyophilized, dissolved in 0.1 M acetic acid, and subjected to HPLC analysis.

2.4. Experimental procedure

After an acclimation period of 72 h, a small blood sample was collected from all individuals between 0900 and 1200. Similar to the sampling in field conditions, the exact time between entering the experimental room (4 rooms with separate entrances) and drawing a blood sample was noted to correct for a potential effect of sampling/handling time on circulating IGF-1 levels. However, handling time was not related to IGF-1 levels ($t = -0.785$, $p = 0.434$). Samples were immediately stored in a fridge until further processing. Subsequently, the IGF-1 treatment (IGF-1) or the control (C, dispersion medium without IGF-1) were applied. Birds were randomly assigned to treatments and each cage contained individuals from both treatment groups. To apply the exogenous hormone manipulation, a spot in the interscapular region was cleaned with alcohol swabs (Rosner GmBh, Vienna) and 100 μl of a dispersion containing either microspheres loaded with recombinant human IGF-1 or only the dispersion medium were injected. For the application, syringes without residual volume (B Braun™ Omnifix®-F Spritzen) and disposable sterile needles (21 G, B Braun™) were used. The applied liquid is highly viscous and forms a depot from where the active ingredient is continuously released.

In order to monitor the hormonal changes after the manipulation, blood samples were collected from different birds on different days over a period of 7 days. Thereby birds were grouped into 6 cohorts. Each cohort consisted of 4 birds from the control group and 6 birds from the treatment (IGF-1 injection) group. Starting with the first cohort, a blood sample was collected from a different cohort every 24 h (±1h) until day 6; on day 7 and 16 post-injection a subset of birds was sampled again.

After each sampling unit, the injection site was examined carefully to record any potential inflammation or skin reaction. The liquid distributed evenly under the skin and the birds' movements were not restricted. The skin around the injection site did not show any signs of inflammation or necrotic tissue.

2.5. IGF1-1 assays

Plasma IGF-1 levels were measured by a competitive ELISA at the University of Debrecen described previously in Mahr et al. (2020). Briefly, 96-well microplates were coated at 4 °C overnight with 100 μl of an antibody raised against IGF-1 in rabbits. The coated plate was incubated for 2 h at room temperature with either 20 μl of standard (known concentrations of synthetic chicken IGF-1 in serial dilutions starting at 500 ng/ml) or 20 μl of sample and 100 μl of biotinylated IGF-1 as a tracer. Sample consisted of 10 μl plasma diluted with 10 μl assay buffer. We did not perform any extraction because previous investigations in this species showed that hormone concentrations with or without an acid–ethanol based extraction protocol were positively correlated and showed similar variation in response to experimental treatments (Lendvai et al. unpublished results). After incubation, the microplate was washed three times with 250 μl of PBS containing 0.025 % Tween 20. After washing, 100 μl of streptavidin-horseradish peroxidase conjugate was added to all wells and incubated at room temperature for 30 min. After washing, 100 μl of tetra-methyl-benzidine was added to the wells and incubated for 30 min. The enzymatic reaction was stopped by adding 100 μl of 1 M H_2SO_4, and optical density was measured at 450 nm (reference at 620 nm) using a Tecan F50 microplate reader.

2.6. Statistical analyses

In total samples from 59 individuals were collected and analysed (IGF-1, $n = 36$; Control, $n = 23$). Results from *in vitro* release experiments were analysed using GraphPad Prism 9. All data from *in vitro* release experiments are presented as mean with $n \geq 3$ and ± standard deviation. The release kinetics was determined by fitting the drug release data to the pharmacokinetic models of the first order, Higuchi, Korsmeyer-Peppas and Weibull model (Costa and Sousa Lobo, 2001; Siepmann and Siepmann, 2008). The adjusted coefficient of determination (R^2 adjusted) was calculated to evaluate the fit of the release data to the respective pharmacokinetic model (Costa and Sousa Lobo, 2001).

We used general linear mixed models implemented in the packages `lmerTest` in the R version 'Bird Hippie' (4.1.2) (R Core Team, 2021) to analyse variation in the *in vivo* IGF-1 plasma concentrations, with time as a fixed factor and random intercepts for individual birds to control for the repeated measurements. Within each sampling unit, treatment groups were compared using post-hoc contrasts, as implemented in the package `emmeans` (Lenth, 2022).

3. Results

3.1. Characterisation of microparticles and in vitro release of IGF-1

Microparticles exhibited a spherical shape with a structured surface and a mean volumetric particle diameter D[4;3] of 28.4 μm ± 0.39, a Dx

(90) of 51.3 μm ± 0.85 and a Dx(10) of 10.4 μm ± 0.12. These results were corroborated by the images of the microscopic analysis. An additional SEM analysis of the particles revealed a highly porous particle structure created due to the porogens sodium chloride and sodium succinate during the particle preparation process (Qutachi et al., 2014) (Fig. 1). On the one hand, the porous matrix partly contributes to the burst effect observed in the *in vitro* experiments (Fig. 2) yet on the other hand, they are a good indication that IGF-1 is not extensively degraded during the release process. During PLGA particle degradation, the polymeric backbone is cleaved finally yielding lactic and glycolic acid. If these degradation products are trapped within the particle matrix, the free acids significantly acidify the particle core. This in turn can lead to instability and degradation of the incorporated protein or peptide (Ding and Schwendeman, 2008; Estey et al., 2006; Fu et al., 2000). The presence of a porous particle matrix can partly alleviate this problematic due to a fast efflux of acidic degradation products through the pores into the surrounding medium. Therefore, the porous structure observed in the SEM images nicely indicates the release of intact and active IGF-1. The encapsulation efficiency of IGF-1 into the microparticle matrix amounted to 70–80 % and was calculated in reference to the maximum loading capacity of 20 μg IGF-1 per 50 mg microparticles.

The release experiments revealed an initial burst release within the first 24 h, amounting to around 40 % of the payload, followed by a continuous release of IGF-1 from the microspheres over the next 14 days. After 15 days, no further significant release of IGF-1 was observed (Fig. 2). These results are in accordance with the release patterns previously presented by Meinel et al. (2001). The release kinetics of IGF-1 from PLGA microparticles correlated best with the pharmacokinetic model of Korsmeyer-Peppas and that of Weibull, with an R^2 adjusted of 0.958 and 0.934, respectively. The drug release kinetic that follows the burst release fitted to first-order kinetics as well (R^2 adjusted 0.943), underpinning the gradual, almost linear release of IGF-1 from the particle matrix after the initial burst.

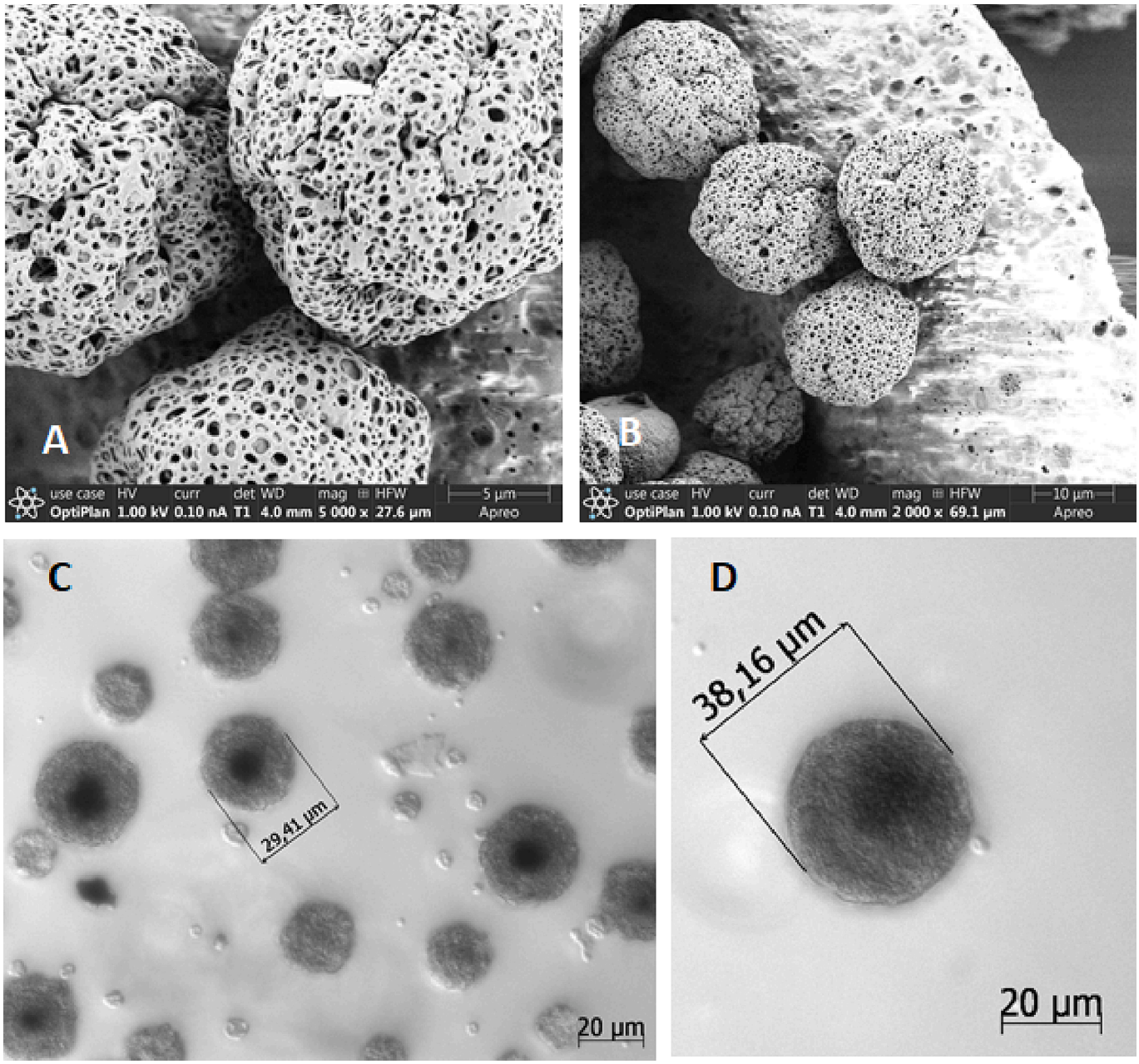

Fig. 1. Scanning electron microscopy images (A and B) and light microscopic images of PLGA RG502H microparticles containing IGF-1 (C and D).

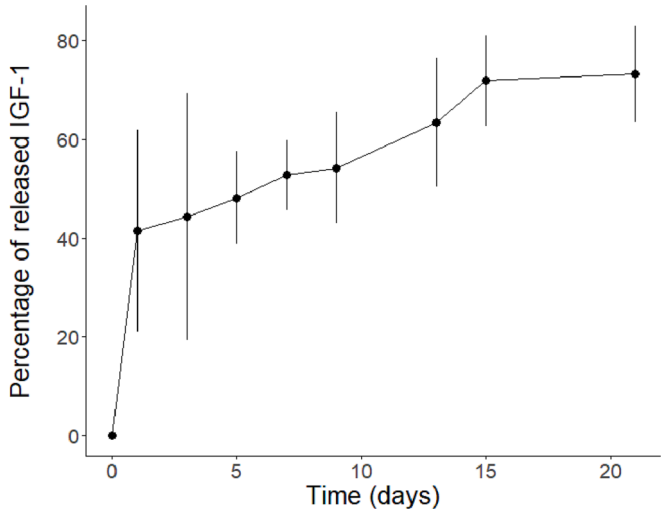

Fig. 2. Cumulative release (mean ± SD) *in vitro* of IGF-1 from PLGA RG502H microparticles over time.

3.2. In vivo release of IGF-1

Before the treatment, there was no difference in IGF-1 levels between the experimental groups (Field: t = 0.65, p = 0.51, day 0: t = 0.63, p = 0.52). According to our expectations, IGF-1 levels increased markedly the day following the injections in the treatment group, but surprisingly, there was also an initial increase in IGF-1 levels in the control group. However, the increase in the treatment group was stronger, resulting in a significant difference between the experimental groups on day 1 (t = 2.21, p = 0.02. Fig. 3). After this initial peak, IGF-1 concentrations dropped in both groups over the following days, and the difference between the treatment and control group remained significant or at the boundaries of statistical significance (day 2: t = 1.65, 0.099, day 3: t = 1.76, p = 0.079, day 5: t = 2.03, p = 0.04, day 7: t = 2.50, p = 0.01). Experimental birds (treatment and control) sampled on days 4 and 6 converged statistically (day 4: t = 1.13, p = 0.26, day 6: t = 0.64, p = 0.51). Finally, by day 16, IGF-1 levels in the treatment group fell back to the levels of the controls (t = 0.44, p = 0.66).

4. Discussion

In this experiment, we tested the suitability of polymeric microparticles as biocompatible vehicles for the elevation of systemic IGF-1 in captive bearded reedlings over a period of 16 days. Our results show that a single subcutaneous injection of IGF-1-loaded PLGA microparticles resulted in an initial spike (i.e., burst release) of IGF-1 with hormone levels remaining significantly elevated for more than 7 - and less than 16 days. Overall, the *in vivo* release dynamics correlate well with the *in vitro* release profile and resemble previous experiments (Lam et al., 2000; Meinel et al., 2001). The *in vitro* release assay involves no degradative processes of the incorporated medical cargo; therefore, a decrease in the IGF-1 concentration would indicate degradation or instability of IGF-1 in the release medium. A rising concentration *in vitro* indicates a release of IGF-1 from the particles. This can be observed until day 15, whereas between day 15 and day 20, there is no further change in the measured IGF-1 concentration *in vitro*; therefore, no additional IGF-1 is released. *In vivo,* the administered IGF-1, however, is degraded and used up over time. If degradative processes are faster than the continuous release from the particle matrix, IGF-1 concentrations decrease *in vivo*. Accordingly, the IGF-1 concentration *in vivo* drops back to baseline levels as soon as no further IGF-1 is released from the particle matrix. A slightly prolonged drug release was yet observed in the *in vitro* setting compared to *in vivo*. This effect has been described previously and is due to faster degradation of the polymers *in vivo* (Cleland et al., 1997; Lam et al., 2000).

Both the *in vivo* and *in vitro* experiments are characterised by an initial burst release within the first 24 h followed by a continuous release over the next two weeks. This heterogeneous release profile hints towards the involvement of different mechanisms in drug release processes from PLGA microparticles (Xu et al., 2017). The disintegration of the particles and the formation of cracks on the PLGA matrix (i.e., bulk-erosion) and the subsequent dissolution of drug molecules near the particle surface explain the initial burst release. This fast drug release is then followed by a period of slower diffusion-based liberation of the encapsulated IGF-1 (Han et al., 2016; Makadia and Siegel, 2011; Meinel et al., 2001). Although, in the present experiment, IGF-1 concentrations remained in the physiological range, as some control birds and individuals before the treatment had high IGF-1 values comparable with the day-1 peak of treated birds, the question arises how an initial burst release after injection can be avoided in future studies. This is an unwanted side effect of many exogenous hormone manipulations and may lead to an overshoot of the target hormone to levels far above the physiological range (Fusani, 2008; Quispe et al., 2015). Previous experiments on rats (Lam et al., 2000) indicate that this effect can be avoided by initiating a "pre-burst" of PLGA microparticles in the injection vehicle. However, it should be considered that birds have higher body temperatures (~41 °C) than rats (37.5–38.5 °C) (Gudjonsson, 1932) and it has been shown that the release kinetics of the polymers are

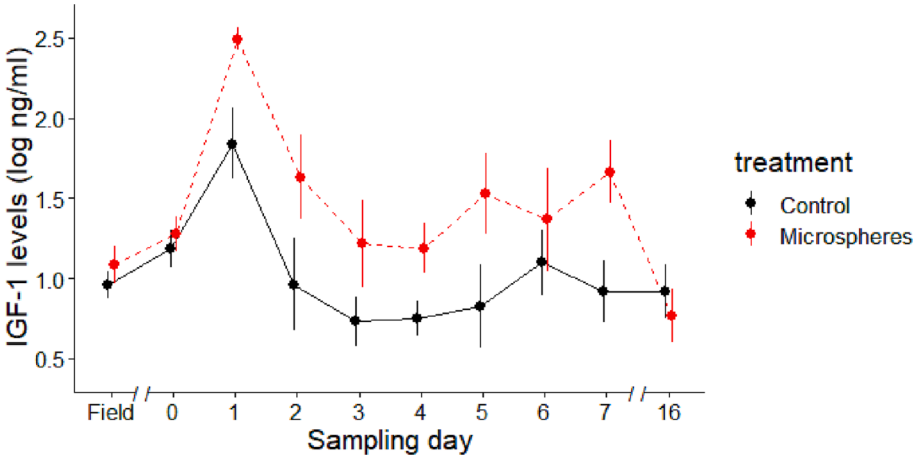

Fig. 3. A single subcutaneous injection of IGF-1 loaded PLGA microparticles (day 0) resulted in a sustained elevation of circulating IGF-1 concentrations in bearded reedlings. Means ± standard errors of log10-transformed IGF-1 values are shown.

sensitive to temperature. Hence, this process might not be as effective for avian species and only lead to faster depletion of the encapsulated peptide.

In the *in vivo* experiment, we observed differences in IGF-1 levels between the control and treatment birds up to day 7, albeit on days 4 and 6 post-injection, the difference between the treatment groups became non-significant. This result is consistent with Lendvai et al. (2021), where IGF-1 concentrations were significantly elevated 24 h after the microsphere injection but became similar to the control group by day 4. While there may be a decrease in the hormone-releasing efficiency around day 4, this decrease may also be due to the initially larger variance of release kinetics at this stage, which also becomes visible in the *in vitro* study (Fig. 2). Our results also show that even if there is a genuine decrease of IGF-1 at this stage, it is temporary, as by day 5 and 7 the IGF-1 concentrations differed significantly between the control and treatment groups (Fig. 3). However, despite this pattern, a clear trend indicates that overall, IGF-1 levels remained higher in the experimental than in the control group (Fig. 3). While these differences are sometimes marginally non-significant, this is most likely due to low statistical power resulting from repeated sampling, the small number of individuals we sampled each day, and the statistical correction for a higher number of multiple comparisons. When we pooled two consecutive sampling days following day 1, the overall differences between IGF-1 and control birds remained statistically significant throughout the first week up to day 7 (day 2–3: $p = 0.02$, day 4–5: $p = 0.03$, day 6–7: $p = 0.02$).

An unexpected result showed a pronounced peak in IGF-1 levels on day 1 post-injection in both experimental groups (i.e., also control birds). While this effect resembled the findings of Lendvai et al. 2021, the observed elevation was more pronounced here. All birds were captured on day 0, and after an initial blood sample, they were injected with the respective treatment (control/IGF-1) and measured for various metrics (not included in this study). This resulted in multiple handling events and an overall longer procedure. On subsequent days, only a subset of birds was captured and apart from a rapid blood sample, only body mass was recorded. Therefore, this initial spike at IGF-1 levels might be the carry-over effect of the arguably stronger disturbance (e.g., long handling time) on the previous day. IGF-1 levels have been shown to decrease due to stressful stimuli (Tóth et al., 2018; Vágási et al., 2020; Valenzuela et al., 2018), but there is strong indication that IGF-1 levels increase over the long term to minimise detrimental effects of chronic stress (McCormick et al., 1998; Xin et al., 2018). This effect might explain the initial increase of IGF-1 in control birds. IGF-1 is also known to regulate immunity, wound healing and inflammatory processes (Bos et al., 2001; Emmerson et al., 2012; Semenova et al., 2008). Hence, an alternative explanation for the temporary increase of IGF-1 might be due to the injection itself. During the experiment, we controlled the injection site daily to record any adverse effects of the procedure. Even though no inflammation or necrotic tissue were detected, the injection of IGF-1 loaded PLGA microparticles or dispersion medium itself might cause a temporary irritation at the injection site (Koutsos and Klasing, 2001). In conclusion, it is highly likely that both the effects of blood collection and handling on the physiological stress response and the local immunological processes that may have been triggered by the injection could be responsible for the observed pattern.

Some techniques for exogenous hormone manipulations (e.g., silastic tubes) allow a continuous release of a target hormone for extended time (up to 4 weeks) (Fusani et al., 2005; Ovid et al., 2018), but in this study, we have only achieved an increase of systemic IGF-1 levels for a period of at least 7, but less than 16 days. However, non-biodegradable pellets or silicone tubes require surgery to replace the vehicle after depletion (Ovid et al., 2018). In this respect, the use of biocompatible polymeric microparticles seems advantageous, as they require only an additional subcutaneous injection to achieve a prolonged release. When wild animals cannot be easily recaptured, adjusting the properties of the PLGA microparticles (e.g., size or resomer type) may offer a possibility to modify the release kinetics and achieve even longer-lasting hormone treatments (Busatto et al., 2018; Matejkova and Podhorec, 2019; Patel et al., 2021; Sun et al., 2008). One potential downside, is that the continuous and prolonged hormone release achieved by the injection of hormone packed PLGA-microparticles is not always suitable or sufficient for mimicking physiological conditions as the levels of some hormones (e.g., corticosteroids or GnRH) undergo fluctuations (e.g., pulsatile release).

5. Conclusion

Traditional methods of field endocrinology might face some limitations as they i) often do not facilitate the sustained long-term release of a given hormone (e.g., injections), ii) require invasive application procedures that include at least one surgery under anesthesia (e.g., silastic tubes) or iii) are restricted to few hormones with lipophilic properties (e.g., beeswax pellets). In this study, we showed that the application of PLGA microparticles loaded with the peptide hormone IGF-1, resulted in a continuous release of the target hormone for several days and potentially weeks after a single subcutaneous injection. In addition, we did not observe any adverse effects, such as inflammation or necrotic tissue on the injection site or note any losses of birds within the course of the study. PLGA microparticles are extremely versatile and their use in a clinical context as a drug delivery carrier demonstrates that they are suitable for the delivery of a variety of substances (e.g., steroid hormones) with different chemical properties. In view of this and our results, this technique appears as a highly promising resource that will open new possibilities for field studies in physiology and behaviour.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author contributions

KM, ÁZL and MA conceived and conducted the experiment, KM, AH, JS and MA collected the samples and the data, ÁZL and MA measured

the samples, FG and ÁZL contributed reagents, ÁZL, MA and GS analysed the data, KM, ÁZL and MA wrote the article, with significant contribution from JS, FG, AH and HH. All authors contributed critically to the drafts and gave final approval for publication.

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