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VILNIUS UNIVERSITY

Vaida Survilienė

Social development and changes in steroid hormones during early ontogenesis of grey seals (*Halichoerus grypus*)

DOCTORAL DISSERTATION

Natural Sciences,
Biology (N 010)

VILNIUS 2022

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Vaida Survilienė

Pilkųjų ruonių (*Halichoerus grypus*) socialinės elgsenos vystymasis ir steroidinių hormonų pokyčiai ankstyvosios ontogenezės laikotarpiu

DAKTARO DISERTACIJA

Gamtos mokslai,
Biologija (N 010)

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LIST OF ABBREVIATIONS AND DEFINITIONS

11-deoxyCOR – 11-deoxycortisol (ng/ml) analyzed with UPC_2-MS/MS
17α-OHP - 17α-hydroxyprogesterone (ng/ml) analyzed with UPC_2-MS/MS
A5 – androstenediol (ng/ml) analyzed with UPC_2-MS/MS
AN – androstenedione (ng/ml) analyzed with UPC_2-MS/MS
CB – plasma cortisol (ng/ml) analyzed with ELISA
COR – cortisol (ng/ml) analyzed with UPC_2-MS/MS
CORNE – cortisone (ng/ml) analyzed with UPC_2-MS/MS
COS – corticosterone (ng/ml) analyzed with UPC_2-MS/MS
CR – social play contact rate (number of play interactions per group size)
CS – saliva cortisol (μg/dl) analyzed with ELISA
DHEA – dihidroepiandrosterone (ng/ml) analyzed with UPC_2-MS/MS
DHT – 5α-dihydrotestosterone (ng/ml) analyzed with UPC_2-MS/MS
DOC – 11-deoxycorticorticosterone (ng/ml) analyzed with UPC_2-MS/MS
E1 – estrone (ng/ml) analyzed with UPC_2-MS/MS
EB – plasma 17β-estradiol (pg/ml) analyzed with ELISA
EB_RIA – plasma 17β-estradiol analyzed with RIA
ELISA – enzyme immunoassay
ES – saliva 17β-estradiol (pg/ml) analyzed with ELISA
KetoTS – 11-ketotestosterone (ng/ml) analyzed with UPC_2-MS/MS
P4 – progesterone (ng/ml) analyzed with UPC_2-MS/MS
PI – social play interaction
PREG – pregnenolone (ng/ml) analyzed with UPC_2-MS/MS
RIA – radioimmunoassay analysis
T2 – testosterone (ng/ml) analyzed with UPC_2-MS/MS
TB – plasma testosterone (ng/ml) analyzed with ELISA
TB_RIA – plasma testosterone analyzed with RIA
TS – saliva testosterone (pg/ml) analyzed with ELISA
UPC_2-MS/MS – ultraperformance convergence chromatography tandem mass spectrometry
Nutritional stages – consisting of two main nutritional periods – suckling (S), lasting around 18 days, and post-weaning or fasting (W), lasting up to 40 days. These are also divided into 4 distinct stages:

S1 – early suckling stage
S2 – late suckling stage
W1 – early post-weaning/ fasting stage
W2 – late post-weaning/ fasting stage

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INTRODUCTION

Grey seals (Halichoerus grypus) are large marine top predators whose population size is indicative of the health status of the wider marine environment (Bäcklin et al., 2011; Gårdmark et al., 2012; Jüssi et al., 2008; Österblom et al., 2007). It is also the largest and most abundant of the three Baltic sea seal species regularly observed on the Lithuanian coast and listed as protected under Lithuanian law (Balčiauskas, 2021). Like other marine mammals, grey seals are prime sentinel species providing early warnings on potential negative natural and anthropogenic impacts on aquatic ecosystem and permitting better management of these impacts that affect both human and animal health (Baily et al., 2016; Bossart, 2011; HELCOM Red List Marine Mammal Expert Group, 2013; Watkins et al., 2022). The population of grey seals is now rapidly growing and has a large impact on the marine economies of Northern Atlantic and Baltic countries (Harding et al., 2007; Svensson et al., 2011). However, grey seals are still affected by a number of environmental factors, such as urbanization, underwater noise, fishing effort and depletion of nutrient resources, and climate change, which is reducing ice cover at sea and forcing seals to breed on land (Bowen, 2016; Hastie et al., 2014; Meier et al., 2004), which leads to higher aggression (Bishop et al., 2015b) and lower pup survival rates (Jüssi et al., 2008). Therefore it is important to identify physiological factors that contribute to individual energy balance, health and behavior, which underpin variability in vital rates and demographic parameters, to help understand the growth and health of grey seals as sentinel species worldwide (Benoît et al., 2011; Donoghue and Boutin, 1995; Hall et al., 2001; Haller et al., 1996; Lang et al., 2011; Pavitt et al., 2014; Vincent et al., 2005).

Social play is considered one of the most important behavior types during early development of mammal species (Burghardt, 2005; Pellis et al., 2010; Pellis and Iwaniuk, 1999). Animal play is described as a voluntary, repeated, not entirely functional behavior that is different from various forms of "serious" behavior in its structure, context and ontogeny, which starts when animal is relaxed in a non-stressful environment (Bekoff and Byers, 2004; Burghardt, 2005). Play is one of the main prerequisites to improve physiological and social experience in many mammal species. Additionally, besides the promotion of cohesion between individuals and training of motor functions, social play also affects cognitive and social skills through the development of key brain areas,

such as the medial prefrontal cortex. In his Surplus Resource Theory of Play, Burghardt (2005a) described several conditions/ surplus resources: ecological, socio-psychological, energetic, and ontogenetic that might be necessary for play to occur (Burghardt, 2010, 2005). The latter is often described as a prolonged parenting period that provides energetic resources and protection for predators for young individuals during social play interaction. However, the parental provisioning is extremely limited during the early developmental period of grey seals. Grey seal social behavior, especially the behavior of young individuals outside the breeding season has been very little studied (Wilson, 1974). In addition, there is almost nothing known about the factors that affect their social play.

Fish-eating aquatic mammals may be extremely vulnerable to endocrine disruptors (EDs) – molecules that mimic or block hormonal activity (Fossi and Marsili, 2003). These endocrine disruptors act by mimicking various natural hormones, mostly steroids, by binding to hormone receptors or influencing cell pathways. Steroids are active lipophilic hormones that produce similar effect in all mammal species and influence social behavior, including social play (Auger and Olesen, 2009; Meaney and Stewart, 1981; Olesen et al., 2005; Ruiz-Cortes, 2012; Wallen and Baum, 2002). Steroid hormones are important for neural development (Semaan and Kauffman, 2010), sexual maturity (Ruiz-Cortes, 2012), immune system function (Klein, 2000) and behavior (Adkins-Regan, 2007) in all mammal species, and has a special priority for energy balance (Katsu and Baker, 2021), nutritional and stress status (Bryan et al., 2015) and behavior. Therefore measurement of steroid hormones allows assessment of sexual maturity and breeding in wildlife (Carlitz et al., 2019; Wilson and Davies, 2007). Although sex steroid levels in the circulation are lower in pre-pubertal individuals than adults, they are still detectable and important mediators of behavior and energy partitioning, especially during early postnatal life (Bell, 2018). Therefore, it is important to know the role of these hormones during early development. However, levels and dynamics of steroids first have to be determined in young individuals. Exposure to high levels of steroidal hormones during critical early developmental period disrupts normal endocrine function and decreases fertility in mammals including humans and can even be transmitted to following generations (Uzumcu et al., 2006; Zubeldia-Brenner et al., 2016).

Measuring steroid concentrations in young animals will help to elucidate the impact they have on the early physiological and behavioral development of grey seals, and whether they contribute to the observed sex-specific

differences in the first-year survival (Hall et al., 2001) and behavior in this species (Breed et al., 2009; Carter et al., 2017, 2019; Robinson et al., 2015; Survilienė et al., 2016; Trippel et al., 1996; Twiss et al., 2012b). While cortisol has been measured in young weaned grey seals in the context of stress and energy balance (Bennett et al., 2013, 2012; Nordøy et al., 1990), there have been no studies on glucocorticoids in suckling grey seal pups. In addition, no studies have investigated androgens and estrogens in juveniles of this species.

Main aim

This work aimed to study the changes in the social behavior and the concentrations of steroid hormones, as potential factors affecting the behavior, during the early ontogenesis of grey seals (*Halichoerus grypus*).

Main tasks

1. To investigate the sex and age of playing individuals and the structure of social play.
2. To investigate how play behavior relates to the size and composition of the group of grey seals.
3. To assess the suitability of saliva for analysis of steroid hormones.
4. To assess performance of commercially available ELISA kits for saliva and plasma measurements, compare the resolution of two matrices and investigate the comparability of plasma steroid concentrations between several analysis methods.
5. To examine the interactions between grey seal pup behavior and steroid concentrations during suckling and post-weaning fast periods.

Scientific novelty

1. Elements of social play in grey seal pups with temporal changes during group formation outside the breeding season were identified.
2. Concentrations of sex steroids of suckling and weaned grey seal pups were measured for the first time by using different methods.
3. Saliva as a matrix for analysis of steroids was applied for the first time in grey seal pups, however the method is limited to the captivity settings and limitedly suitable to be used in the wild.
4. Typical glucocorticoid concentration values of grey seals during early development were obtained by using multiple analysis UPC_2-MS/MS method and typical values reported for the first time.

5. Relationship between glucocorticoids (11-deoxycortisol, corticosterone, 11-deoxycorticosterone) and the social behavior of grey seal pups was investigated for the first time during suckling and post-weaning fast periods.

The relevance and practical importance of the research

6. Detailed ethogram for grey seal social play behavior was developed and can be further applied with or without modifications for the investigation of social play of grey seals in other contexts.
7. The limited suitability of saliva for steroid research in the wild was shown that should discourage the use of this method with wild animals in the future.
8. Possibility to use saliva as a matrix for investigation of steroids, such as estradiol, cortisol and possibly progesterone, in captivity with trained grey seals and potentially other pinniped species was demonstrated.
9. Problems with commercially available ELISA kits and extraction methods that require pre-investigation in separate laboratories were identified.
10. Sensitive UPC_2-MS/MS method for simultaneous detection of multiple steroids that can be used for detection of steroids in grey seals and other marine mammals in the future was developed.
11. Typical levels and variation of steroid hormones in grey seal pups that could be used as a reference for future investigation were estimated.
12. The associations between steroid hormones and pup behavior obtained during pilot studies provide an opportunity for further investigation of these processes in the future.

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1. SOCIAL PLAY BEHAVIOR OF GREY SEALS DURING THE NON-BREEDING SEASON

1.1. LITERATURE REVIEW

1.1.1. Definition, costs and benefits of the social play

Animal play is described as a voluntary, repeated, not entirely functional behavior that is different from various forms of "serious" behavior in its structure, context and ontogeny (Burghardt, 2005). It starts when animal is relaxed in a non-stressful environment. Play is one of the main prerequisites to improve physiological and social experience and is usually divided into three separate categories that include solitary locomotor-rotational, object and social play. It is assumed that first two are more related to the development of motor-neural skills and help to improve movement and coordination. Social play is considered to lead to more complex group activities (Graham and Burghardt, 2010). Social play has an important role in the behavioral development of juveniles in many land mammals (e.g., wolves [*Canis lupus*; (Bekoff, 1974a)], rats [*Rattus sp.*; (Pellis and Pellis, 1998)], spotted hyenas [*Crocuta crocuta*; (Drea et al., 1996)], and brown bears [*Ursus arctos*; (Fagen and Fagen, 2004)]), as well as in many other polygynous pinniped species (e.g., South American fur seals [*Arctocephalus australis*; (Harcourt, 2010)], Galapagos fur seals [*Arctocephalus galapagoensis*; (Arnold and Trillmich, 1985)], Steller sea lions [*Eumetopias jubatus*; (Gentry, 1974)], and elephant seals [*Mirounga angustirostris*; (Reiter et al., 1978)]).

There are proximate and distant causes that explain why animals play. As for proximate causes, animals play because it is a rewarding and pleasurable activity (Balcombe, 2009; Trezza et al., 2010; Vanderschuren et al., 1997), also initiated by stress-free environment with sufficient energy resources (Almeida et al., 1996; Almeida and Araujo, 2001; Arnold and Trillmich, 1985) or boredom (Graham and Burghardt, 2010) and necessary for the growth of specific prefrontal brain areas during critical developmental periods (Bell et al., 2010). Social play is necessary for successful neural, cognitive, social and sensimotor development (Fone and Porkess, 2008). Deprivation of the social play may lead to difficulties coping with the future social behavior (Hol et al., 1999). Social isolation during the play sensitive period leads to hyperfunction of the hypothalamus – pituitary – adrenal (HPA) axis in rats and increased emotional reactivity to stress, especially in males (Lukkes et al., 2009; Weiss et al., 2004).

The social play period in rats coincides with formation of the medial prefrontal cortex (mPFC), which is associated with planning, motivation, motor habits and behavioral models. Social play helps to develop mPFC through simplification of dendritic structures and modification of neurochemical pathways (Baarendse et al., 2015; Bell et al., 2010). Therefore, during social play through trial and error an animal develops behavioral models that they use in the future. In other words, the animal gains social experience and in polygamist pinniped experience is related with reproductive success such as in Weddell seals (*Leptonychotes weddellii*) (Harcourt et al., 2007) and sometimes is more important to gain access to females than energetic resources (Lidgard et al., 2012).

Distant causes of play explain social play as an adaptation factor of young individuals that facilitates social integration of young individuals because play helps them practice and dynamically assess their physical capabilities under nonstressful conditions, develop fighting skills, and form dyadic or group relationships (Burghardt, 2005; Fagen and Fagen, 2004). Adult rats deprived of social play (mainly play fighting) during their infancy become hyperdefensive during social contact: they are not able to exhibit proper submissive behaviors towards dominant males or coordinate movements with other mates, are overly stressed, and escalate aggression (Hol et al., 1999; Pellis et al., 2010). Improper behavior towards conspecifics does not allow maintaining of stable relationships within a group and might have implications for reproductive success (Panksepp and Beatty, 1980; Trova et al., 2021).

In his Surplus Resource Theory of Play, Burghardt (2005a) described several conditions/ surplus resources: ecological (i.e., favorable environmental conditions with intense competition among rivals), socio-psychological (i.e., need for stimulation and a complex adult behavior in the future), energetic (i.e., possibility to thermoregulate and easily recover from vigorous activity), and ontogenetic (i.e., long juvenile period) that might be necessary for play to occur (Burghardt, 2010, 2005). Social play is a costly behavior for young individuals not only due to high metabolic rates but also because young individuals are less vigilant during play activities and become more vulnerable to predators or might suffer accidental injuries (Bekoff and Byers, 2004). An extreme example given by (Harcourt, 1991) showed that 22 of 26 South American fur seal (*Arctocephalus gazella*) pups that were caught and killed by southern sea lions (*Otaria byronia*) had been involved in social play. Playing pups were distracted, moved away from their mothers, or ignored fleeing group members. Since play behavior made up only about 6% of their total time budget, it was a very costly behavior in terms of surviving. In this case, the lactating female South American fur seals alternate

periods of suckling the pup with periods of feeding at sea; therefore, there are times when pups are left onshore unattended. Therefore, prolonged parental provisioning of young individuals (which leads to an extended juvenile period) is an important prerequisite for play behavior in mammals that helps to reduce their survival costs (see Burghardt, 2005). However, in cases where parental provisioning is limited or terminates early, other group members could act as an "alarm signal" when necessary and provide direct or indirect protection. Indeed, in many groups of mammals, play behavior is positively related to group size (Burghardt, 2005). Juveniles of gregarious ungulates and primates tend to play more frequently and with longer durations when group size is larger (Baldwin and Baldwin, 1974; Berger, 1979).

1.1.2. Social behavior of grey seals

Grey seals (*Halichoerus grypus*) are gregarious, polygynous, sexually dimorphic mammals (Boness and James, 1979). Male grey seals become physiologically mature at approximately 3 to 5 years of age as do females, but most males do not take an overt part in breeding colony matings until they are at least 8 years old (Hall and Thompson, 2009). This delay in reproductive activity is likely because young mature males must gain mass and social experience to be able to compete with other adult males (Anderson and Fedak, 1985; Boness and James, 1979; Harcourt et al., 2007; Lidgard et al., 2012, 2005).

Although few studies described grey seals to be social and playful animals outside the breeding season (Hunter et al., 2002; Schusterman et al., 1970; Wilson, 1974), it is not easy to observe their social play behavior in the wild during the non-breeding season since, as mentioned before, they are typically found far from the coast either in the water or on remote islets or sandbars. Therefore, taken together, environmental conditions (i.e., rain, fog, changing water level, and waves), the instability of aggregations, the typically large haul-out distance from the mainland, and the aquatic lifestyle of grey seals make observations in the wild during the nonbreeding season hard to plan and difficult to perform (Mellish et al., 2006). Therefore, most behavioral observations occur during the breeding season when groups of grey seals are more stable and easier to observe, but leaves a huge knowledge gap about their social behavior outside the breeding season (Lidgard et al., 2001; Pomeroy et al., 2000; Ruddell et al., 2007; Twiss et al., 2007, 2012a).

Grey seals correspond positively to the conditions described in a Surplus Resource Theory of Play (Burghardt, 2005; Graham and Burghardt, 2010),

including energetic resources, save environment to perform activities, complex behaviors of adult males during the breeding season that must be practiced and considerably late social maturity. However, differently from many other pinniped (German Riet Sapriza, 2020) and terrestrial mammal species (Bekoff, 1974b; Burghardt, 2005; Gard and Meier, 1977), the parental provisioning is extremely limited during their early developmental period. In grey seals, a short 17 to 21 day lactation period is followed by abrupt weaning, and no further parental provisioning is provided (Mellish et al., 1999a; Pomeroy et al., 1999). Grey seal pups move very little during lactation and spend most of their time resting, presumably to avoid loss of contact with the mother and ensure they use as much of the energetic sources they are getting during suckling for growth, as larger pups have more chances to survive their first year of life (Hall et al., 2001; Pomeroy et al., 1999). During suckling period, pup play behavior becomes more evident with age, but is usually solitary and not directed towards other seals (Kovacs, 1987a).

Play behavior is most easily observed while grey seals are on land. In common with other pinniped species, grey seals use remote islands or sites exposed by low tide to come ashore between foraging trips out at sea. These periods ashore, haulouts, often occur within a limited space such as a rocky islet or tidal sand bank. Telemetry studies indicate that grey seals tend to spend approximately 40 to 75% of their time in the water near haul-out sites (McConnell et al., 1999; Sjöberg and Ball, 2000). Grey seals are also known for having strong site fidelity to their specific haul-out sites during both nonbreeding (Karlsson et al., 2005; McConnell et al., 1999; Sjöberg and Ball, 2000) and breeding (Pomeroy et al., 2000; Twiss et al., 1994) seasons. When they are not breeding, grey seals haul out to molt once a year, maintain effective thermoregulation, rest, and possibly digest (Riedman and Boeuf, 1982). It is considered that the "cost of immersion" motivates pinnipeds to haul out for rest after foraging (Watts, 1996); and if they are deterred from hauling out, they spend more time ashore subsequently than expected (Brasseur et al., 1996). Grey seals haul out to form large groups that are sometimes close to mainland beaches, where they can be vulnerable to danger because of reduced mobility on land. Thus, gregarious behavior at haul-out sites may afford increased vigilance and help with the detection of potential threats (Da Silva and Terhune, 1988; Terhunet and Brilliant, 1996; Watts, 1996).

McConnell et al., (1999) suggested that the large amount of time spent near haul-out sites, especially for young individuals, might be related to social

interactions. Therefore, young individuals playing in a mixed age group obtain public information from the reaction of adults to possible dangers.

Grey seals are often observed playing, but very few studies have been performed to investigate this behavior (Hunter et al., 2002; Wilson, 1974). Recent growth of the grey seal population in the North Sea has helped focus attention on understanding the structure of the population, including group behavior and the behavior of separate individuals (Bowen, 2016).

1.1.3. Rationale

Social play is considered as one of the most important social activities between weaning and puberty in mammals (Bekoff, 1974a; Bell et al., 2010; Ravn et al., 2011; Thor and Holloway, 1984). As discussed previously, it not only helps to develop practical motor skills, but is also associated with enhanced specific cognitive and social functions. Social play is very little studied in grey seals (Wilson, 1974). Studies show that newborn grey seals spend a majority of the time resting with very limited social interactions, the majority of which take place between a female and a pup (Kovacs, 1987b). At the same time, almost no observations of social play were performed outside the breeding season (Wilson, 1974), therefore there is a whole large gap of limited knowledge of social behavior of grey seals starting with a post-weaning fast to adulthood (Fig. 1.1). In our study we sought to investigate social play in grey seal colonies outside the breeding season.

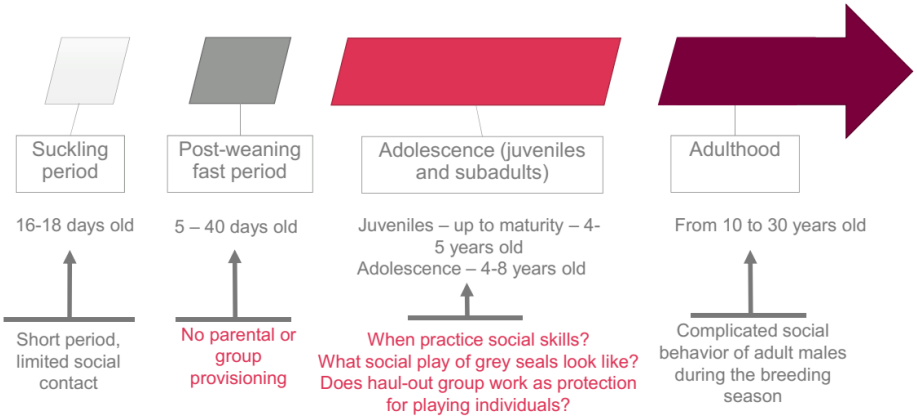


Fig. 1.1. Schematic view indicating critical social development stages, their approximate duration and the knowledge about key social behavior during these stages.

Two hypotheses were raised for this project: a) if social play is important to train for future behavior, play fight and mating behaviors should be dominating during the social play of grey seals; b) if a haul-out group is a mediator for playing individuals that indicates a safe environment to haul-out, play activity should be positively related to the group size during the haul-out formation period.

Main aim:

To investigate the social play behavior of grey seals (*Halichoerus grypus*) and the factors affecting the social play rate outside the breeding season.

Main objectives:

1. To investigate the sex and age of playing individuals,
2. To investigate the structure (behavioral elements) of social play,
3. To investigate how behavior relates to the size (i.e., number of individuals) and composition of the group of grey seals,
4. To investigate the temporal sequence of play during observations of haul-out groups.

1.2. MATERIALS AND METHODS

1.2.1. Study location and animals

The study was performed in Tentsmuir National Nature Reserve on the east coast of Scotland (56.43, -2.80). Abertay Sands are exposed at the mouth of the River Tay during low tide (Fig. 1.2a and b). The tidal sand banks were the haul-out sites used by grey seals. Although a few harbor seals (*Phoca vitulina*) sometimes hauled out on the mainland beach, grey seals rarely did this.

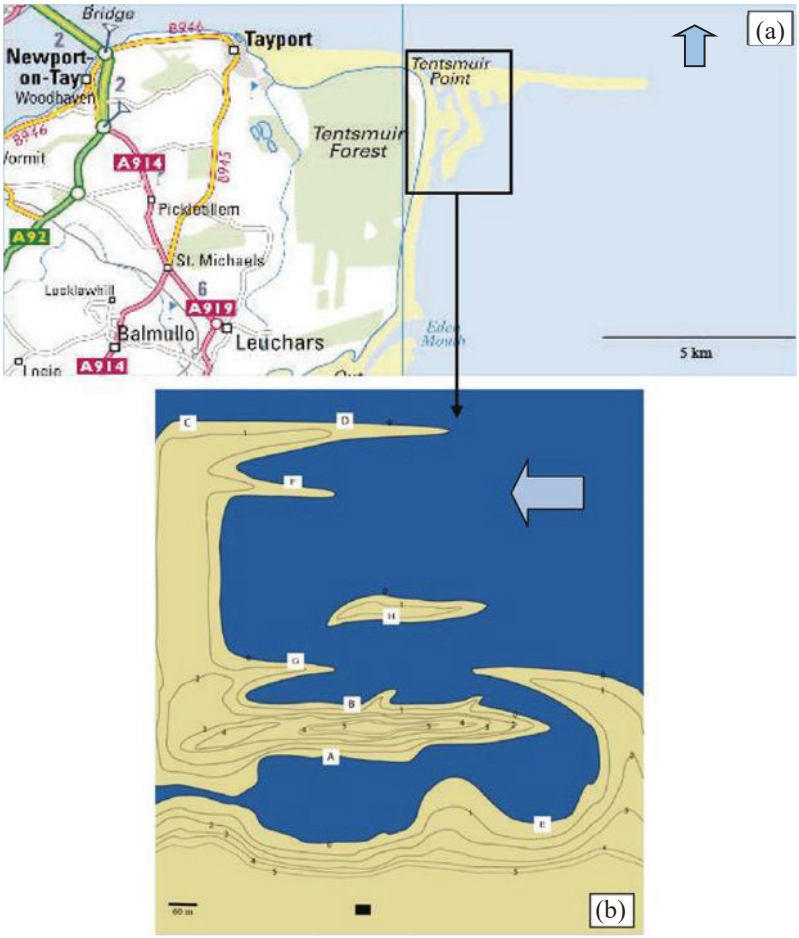


Fig. 1.2. (a) Location of the study area (www.streetmap.co.uk); and (b) schematic overhead view of the haul-out area. Annotated lines show approximate water level, and numbers denote hours before/after low tide (0) when the proportion of sand (light brown) above the water (blue) reaches its maximum. Haul-out areas used by groups of grey seals (*Halichoerus grypus*) are shown (A-H). The solid black rectangle shows the approximate position of the hide from which observations were taken.

1.2.2. Age and sex determination for *ad libitum* sampling

Grey seals present were classified into age and sex categories according to visible features, including genital openings when visible, body size, development of secondary sexual features (e.g. nose length, distance between eyes, neck thickness, etc.), and pelage marking dimorphism (Davies, 1957; Hewer, 1964). Genital openings were visible around 20 % of time (see Kenyon and Maxwell, 1969). If genitalia were impossible to observe, sex was assigned based on the pelage pattern and secondary sexual characteristics (around 80 % of all cases). The entire background of grey seal males is formed by a dark shade (i.e., brown, black, and grey) and punctuated by darker irregular spots (Table 1.1). The most distinctive features of mature males were secondary sexual characteristics, including a large neck and shoulders (often scarred) and a long nasal rostrum (Boness and James, 1979). Adult males are, in general, larger than adult females, reaching a body length of 250 cm and a mass of 350 kg. In contrast, females usually grow up to 200 cm long and weigh up to 250 kg (Bowen, 2016). Typical female pelage has a light grey background with dark irregular patches overlaid (Hall and Russell, 2018) (Table 1.1).

Three age groups were distinguished: (1) adults, (2) sub-adults, and (3) juveniles. Males with bulky and rugose necks and shoulders and with a prominent rostrum were classified as adults (M A). Males were considered to be sub-adult (M Sub) when they had a smaller body mass in comparison, leaner neck, and fewer scars on their neck, indicating that they had been involved in few or no fights (Table 1.1, Fig. 1.3). Females that were obviously pregnant (i.e., with a distended abdomen) and had a typical light pelage were considered to be adults (F A), whereas sub-adult females (F Sub) had a flatter abdomen and were relatively smaller. All small individuals without well-defined secondary sexual characteristics but with clear pelage markings were classified as juveniles (Juv) (King, 1983). Sub-adults and juveniles altogether are also referred as young individuals in the text. In most cases, the sex of juveniles was not determined, thus we did not ascribe sex for this age class of grey seals. If it was impossible to determine the age and sex of an animal, it was considered unknown (Unkn). Unknown animals were typically adults or sub-adults.

Table 1.1. Visual indicators to discriminate individuals of different age and sex.

	**Adult male** Inter-sex specific characteristics: 1. Large in size. 2. Long hooked nose. 3. Dark and more uniformed pelage. Age specific intra-sex characteristics: 1. Large rugose neck 2. Feet are relatively smaller comparing to the body size. Author: Fisheries and Oceans, Canada, W. D. Bowen https://www.marinespecies.org/photogallery.php?album=680&pic=39229
	**Adult female** Inter-sex specific characteristics: 1. Short and narrow nose. 2. Lean neck. 3. Light pelage with distinct markings. Age specific intra-sex characteristics: 1. Round abdomen indicating pregnancy. 2. Relatively larger in size.

Author: © lookphotos / age footstock, https://www.lookphotos.com/en/images/70150647-Grey-Seal-Grypus-halichoerus-two-sub-adults-play-fighting-in-surf-North-Lincolnshire-UK-November-2005

Sub-adult male (on top)
Sex specific characteristics:

1. Relatively large in size.
2. Longer hooked nose, however not as pronounced as in adults, distance between the eyes is also bigger than in females.
3. Dark and more uniform pelage.

Age specific characteristics:
1. Lean neck without scars.
2. Feet are relatively larger comparing to the body size, showing the tendency to grow.

Sub-adult female (bellow)

Sex specific characteristics:
1. Short and narrow nose.
2. Lean neck.
3. Light pelage with distinct markings.

Age specific characteristics:
1. Abdomen is flat, indicating no pregnancy.

Relatively smaller than other females in size.

Juveniles/ yearlings

1. Smallest individuals in the group.
2. No distinctive sex differences – hooked long nose.
3. Flippers relatively larger to the whole body.
4. The pelage could be pale brown due to an extended period of molting.

© Colin Seddon/Shutterstock

Fig. 1.3. Grey seal sub-adult males initiating social play interactions in the shallow water on the edge of a haul-out group. At least two already hauled-out sub-adult males can be seen to the sides of the interactants (circled in white). Photo by Claire Laicey.

1.2.3. Observation Procedure

Behavioral observations were made from 15 June to 6 August 2009. In total, 107 h of observations were used for the analysis. Observations were made during daylight hours between 0700 and 2100 h BST. The observation time and duration depended on weather conditions and tidal conditions. No observations were made during unfavorable weather conditions when visibility was restricted by fog or when the closest group of grey seals was too far away to be observed in sufficient detail (i.e., farther than approximately 400 m).

Video recordings using a Sony Handycam (DCR-SR36, × 40 optical zoom, × 200 digital zoom, 40 GB internal hard drive) with voiceover were made alongside visual observations. Bushnell binoculars (8 × 32 mm), an Opticron HR80 telescope (× 20 to 60) with a tripod, and data sheets were used for recording data. Observations were made from a wooden hide built on the top of a sand dune on the mainland overlooking the closest haul-out group (or group) of grey seals, 10 m from the high water mark and 200 to 300 m from the closest haul-out site A (Fig. 1.2b).

Ad libitum sampling (Altmann, 1974; Martin and Bateson, 1993) between scans was used for detailed play observations (Fig. 1.3). It was possible to determine the sex of most of the interactants and assign them to a specific age group as described previously. Behavior was recorded according to an ethogram (Table 1.2) based on previous studies of juvenile play behavior of grey seals (Wilson, 1974), adult grey seal observations during the breeding season (Twiss, 1991) and previous observations. Visual examples of behavioral element are provided in Table 1.3. A random dyad of playing individuals would be chosen and observations of behavior of both individuals would start immediately. A total of 84 PIs out of 2425 behavioral elements were recorded during the *ad libitum* sampling (1218,44 min. or 20,31 hours). Behavioral element is the smallest distinguishable and describable behavioral unit, described in this study ethogram (Table 1.2). PIs were identified as two individuals playing or starting to initiate a contact and would be recorded as on the first opportunity. Observations on PI would cease when both individuals moved away from the visibility field in the water or deeper in the colony (60 %), both animals hauled out on land (23,53 %) which would usually terminate in animals resting or performing other non-social types of behavior, or when animals separated when one animal would haul out and another would leave (16,47 %).

Most observations were performed on the margins of the colony. 79,11 % of play behavior elements were observed in the shallow water – area where at least 2/3 of the animal body is still visible, and 20,89 % on land, however still in the periphery of the colony. PIs in deeper waters were not recorded because it was impossible to follow animals throughout the play bout.

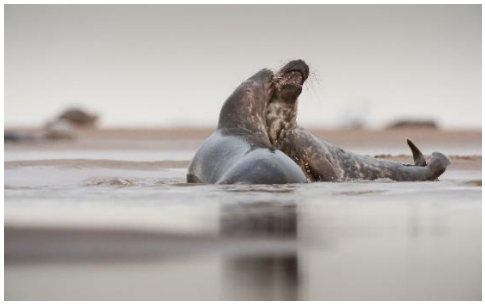
Table 1.2. The ethogram for grey seal behavior observations.

Symbol of the behavioral element	**Explanation of the symbol**
	I. Locomotion – movements to/from a location or another animal.
Ch	Chase – one individual is chasing the other.
Loc A	Locomotion away from other individual;
Loc W	Locomotion towards/to the water;
Loc L	Locomotion towards/on land.

Symbol of the behavioral element	Explanation of the symbol
II. Initiation – behavioral elements that initiated the start of the play behavior or maintained it after it ceased.	
Ap	Approach – the animal appears/emerges moving towards the other.
Mz	Muzzling – one individual touches the other with its whiskers or muzzle.
SI	Social investigation – one individual is attentively looking into the other with the head a little lowered, sniffing other individual.
III. The interruption of the play – behavioral elements that usually interrupted or brought play behavior to an end.	
A	Alert – the state of vigilance when the body of an individual is tensed, neck extended and head erect; animal checks the environment (looks around, sniffs the air and listens).
F	Flippering – one animal touches the other, itself or water/air with its flipper moving vigorously during the play (in other situation sometimes seeking to calm down, show a different direction or expressing agonistic behavior); the behavior last longer than 4 sec.
OMT	Open mouth threat – a threat towards another animal, with whiskers erect and mouth open, sometimes accompanied by vocalizations.
R	Rest – the period of inactivity.
IV. Play behavior – a variety of behavioral elements that were considered as play behavior.	
A. Social play-fight pattern	
BHF	Biting hind flippers of a partner.
OM (Voc)	Open mouth (vocalization) – an animal is holding mouth open, whiskers sometimes erected, towards the playmate, during a PI.
W	Wrestling (Play-fight) – two individuals imitate a fight, without real aggression. Typically involves pushing each other with their necks and flippers, lunging and imitating a bite (Table 1.3a)
Ro W	Rolling in the water – a pair of seals spins around each other in the water, sometimes chasing each other in a very close proximity, sometimes holding each other with front flippers. It could be considered as a play-fight in the water, where movements are not limited by the land and more freedom is for players to change positions.

Symbol of the behavioral element	Explanation of the symbol
L	Lunging – brief direct sharp head movements towards another animal.
	B. Sexual pattern
IM	Imitation of mating – subject mounts another seal from the back, makes repeated pelvic thrusts resembling copulation (but no copulation occurs) and may bite and hold neck skin of the interactant during the process.
Mo	Mounting – one individual mounts the other individual (Table 1.3d).
NB	Neck bite – animal mouths, noses or gently bites the neck of the playmate.
	C. Other playful elements
P	Pinning – one individual goes on top of the other (not from the back) pressing it to the ground. It is usually followed by vocalizations, splashing, and muzzling, sniffing and non-aggressive biting, such as mouthing (Table 1.3b-c).
B	Bite – an animal bites the playmate.
HS	Head shake – vigorous moving of an animal back and forwards and to different directions.
Ro A	Rolling away – rolling away from the other individual.
Ro B	Rolling on the back – an individual rolls on its back (Table 1.3c).
Ro T	Rolling towards other individual.

Table 1.3. Examples of different behavioral elements.

	**a) Wrestling** (Play-fight) © Shutterstock
	**b) Pinning** (sub-adult male on top, sub-adult female below) © lookphotos
	**c) Roll back** (sub-adult female below) and **pinning** (sub-adult male on top) © Dreamstime
	**d) Mounting** (Sub-adult male on top) © Dreamstime

1.2.4. Haul-Out Group Formation and Temporal Frequency of Play

The scan sampling method (Altmann, 1974; Martin and Bateson, 1993) was used:

(1) to obtain information about haul-out patterns in the study area (the number of haulouts and their position in the study area (Fig. 1.2b); the approximate number of individuals in each haulout);

(2) to obtain information about the sex and age structure of the haul-out group of individuals and the number of playing individuals in the closest haulout. The number of grey seals visible in up to eight haul-out sites was recorded every hour from the start of observations (i.e., scans). Groups of grey seals were considered separate if the distance between groups was more than 20 m. Locations of these groups are indicated by letters A through H in Fig. 1.2b.

Detailed observations were restricted to scans from haul-out site A. Scans of this haul-out site were repeated at 15-min intervals. Number of grey seals, their sex and age, and number of social PIs were recorded along with the date and time of day. Only social play was recorded, and only dyadic interactions were seen and recorded during the social play of grey seals. Due to the partially aquatic nature of PIs it was not always possible to assess the sex and age of all playing individuals during scan sampling, due to a nature of scanning quickly through the colony registering number of contacts, therefore the indicator was not used in the analysis for temporal change of CR. Observation scans started as soon as the observers entered the hide.

Water-level fluctuation was a limiting factor for group formation because haul-out groups could be formed only on the sand banks that appeared during the low tide. Observation times were expressed as ***time relative to low tide (TRT)***. Water level and tidal state were registered using the Admiralty Easy Tide website (http://easytide.ukho.gov.uk) predictions for River Tay Bar, Scotland. During the observation period, low tide (i.e., hour 0) water levels dropped to 1,38 m above the chart datum (i.e., meters above a fixed base elevation at a local tide station to which all water level measurements are referred); at high tide, approximately ± 6,18 h from low tide, the water level rose up to 4,65 m above datum (see Fig. 1.2b).

The beginning of the haul-out group's formation was defined as the time point at which the observer could clearly see the process of two individuals hauling out on land. The haul-out formation process was recorded in time

periods (15-min. scans) from the beginning of its formation (given as 0 time). These intervals were combined to 1-h periods in the analysis, and these hourly intervals are further denoted as the ***haul-out formation period (HFP).*** The group size included individuals who hauled out and those who were playing or present in the adjacent shallows.

1.2.5. Statistical Analysis

Data analysis and graphs were prepared using Excel (Microsoft Office, 2018), STATISTICA (ver. 8.0.55, Statsoft Inc., USA) and IBM SPSS Statistics Version 22 (IBM Corp., 2013), with p values considered to be significant based on $\alpha = 0{,}05$.

1.2.5.1. Social play analysis

Behavioral data from *ad-libitum* sampling was used to calculate overall percentage of behavioral repertoire (%), behavioral element rate (element/min.) and proportion of element duration (%) during PI. Overall ***percentage of behavioral repertoire (%)*** is a ratio of all scans of particular behavior and a total amount of scans calculated for each age/ sex group and expressed as a percentage. Behavioral element rate or ***rate (element/min.)*** was calculated by dividing number of scans of particular behavioral element during a particular PI from a duration (min.) of the same PI. Proportion of the element duration or ***proportion of the duration (%)*** was calculated by dividing the duration of a behavioral element during a particular PI from a duration (min.) of the same PI. Median duration (seconds) (25-75 % quartiles) was provided for each element in the text.

The Median test (χ^2) was used to indicate differences of behavioral element ***rate*** (element/min.) (N = 168) and ***proportion of the duration*** (%) (N = 52) of individual grey seals of different sex and age during the social PI.

1.2.5.2. Temporal change of play analysis

Both the absolute number of PIs in the closest haul-out group and CR, or the proportion of playing individuals in a group at a particular time, were used in the analysis. CR was calculated by the formula, where y_i is the number of dyadic PIs and N_i is the group size at a particular observation time (*i*):

Formula 1) $$CR_i = \frac{2 \times y_i}{N_i}$$

Nonparametric statistical analysis methods such as Friedman's ANOVA with Kendall's coefficient of significance ($\chi 2$, Kendall's W) was used to compare proportions of different sex and age individuals in the study haul-out (A) during each scan. The Mann-Whitney U test (U) was used to investigate whether there were significant differences in haul-out group size (HS) and proportion of different sex and age seals when there were no social PIs (PI = 0) vs. at least one PI (PI > 0). Kruskal-Wallis test (H) searched for significant differences within haul-out group size (HS), proportion of different sex and age individuals, contact rate (CR) and the number of PIs during different haul out formation period (HFP). Spearman rank correlation (R) tested the strength and significance of correlation between HFP and GS, proportion of different sex and age individuals, PI, CR; also between HS and CR, PI; between PI and CR; between proportion of different sex and age individuals and PI, CR. Results are presented as median values with quartiles (Q_1-Q_3), except if indicated otherwise.

Negative binomial generalized linear model (NBGLM) with log link was used to investigate factors effecting the number of PIs (*PI*). Negative binomial regression is often used for modeling over-dispersed count variables. The model is described by the simplified formula:

$$PI = e^{(HS+PropF+PropM+PropSF+PropSM+PropJ+ToD+D+HFT+TRT)}$$

where *PI* is a number of PIs observed, *HS* – haul-out size (number of individuals), *PropF* – proportion of adult females (%), *PropM* – proportion of adult males (%), *PropSF* – proportion of sub-adult females (%), *PropSM* – proportion of sub-adult males (%), *PropJ* – proportion of juveniles (%), *ToD* – time of the day, *D* – date, *HFT* – haul-out formation time, *TRT* – time relative to low tide (0), which shows the availability of dry land for haul out.

Best NBGLM model was chosen by removing the statistically insignificant variables from the model and checking the smallest value of Akaike information criterion (AIC). Also omnibus test with likelihood ration chi-square was used to evaluate whether the model is better than an intercept only model.

1.3. RESULTS

1.3.1. Social Play Interactions

A total of 168 individuals were involved in dyadic interactions. Most of them were sub-adult males (61,76 %) and sub-adult females (13,53 %). Less frequent participants were adult males (10 %) and juveniles (9,41 %). There were no adult females seen interacting. There were also seven occasions where sex and age were not identified.

The most frequent interactions were between sub-adult males (42,35 %) and between sub-adult females and males (21,18 %) (Table 1.4).

Table 1.4. PIs between interactants by age and sex.

Ind. 2 / Ind. 1	Sub-adult male	Sub-adult female	Adult male	Juvenile	Unknown	Totals
Sub-adult male	35	18	7	4	4	68
Sub-adult female		0	4	0	1	5
Adult male			2	1	1	4
Juvenile				6	0	6
Unknown					1	1
Totals	35	18	13	11	7	84

The majority of PI records lasted less than 10 min. The median duration of PIs records was 4,52 min. (from 2,5 min. to 47,25 min.). Field notes (totally n = 51 PI recorded) were used to investigate a sequence and consistency of a PI and their recordings were included into the calculation of behavioral rate (elements/min.) and proportion (%) of total amount of play elements, while duration of different elements was investigated only from video recordings (n = 26, total duration – 389,15 min. or 6,49 hours).

17,18 % of behavioral elements recorded during PI belonged to *initiation of play*, 13,81 % – to *interruption of play* and 8,95 % – to locomotion and 60,06 % of behavioral elements during PI belonged to play behavior (Fig. 1.4). Wrestling (W), rolling in the water (Ro W), rolling on the back (Ro B) and mounting (Mo) were the most common social play elements during recorded PIs.

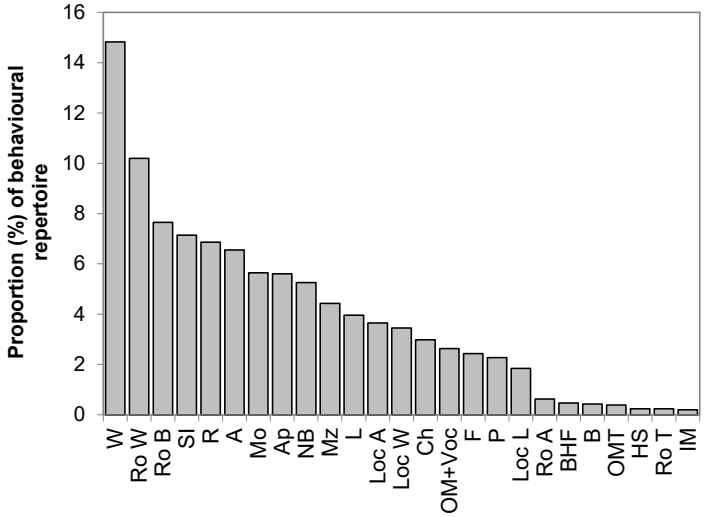


Fig. 1.4. Proportion of behavioral elements (%) during social PIs. Abbreviations explained in Methods section (Table 1.2).

The difference of behavioral rate (behavior/ min.) between different age and sex classes was only significantly different for wrestling (W), flippering (F), locomotion away (Loc A), roll towards (Ro T) and open mouth treat (OMT) (Table 1.5). Sub-adult males had the highest wrestling rate while sub-adult females had the lowest. Flippering was most frequent among adult males and juveniles, similarly adult males and unknown individuals tended to move away more often. Sub-adult males and juveniles tended to roll towards other individual more often. Agonistic interaction, such as open mouth treat, was most frequent among adult males, sub-adult females and juveniles.

Due to a small sample size of behavior duration (Table 1.6), there was a limited possibility for comparative analysis. Only the duration proportion (%) of alert (A) and approach (Ap) behaviors were significantly different between different sex and individuals and in both cases juveniles had the longest duration.

For *M-Sub* and adult males wrestling and mounting were dominating behavioral elements. *M-Sub* were the only ones to perform imitation of mating, while adult males more frequently performed mounting behavior. *F-Sub* would differ in their behavior by more frequently rolling in the water (Ro W) or on the back (Ro B), pinning (P) and neck bite (NB) behavior. They would more often open mouth (vocalize) (OM (Voc)), but were very rarely seen wrestling or mounting. Juveniles predominated in head shaking (HS) behavior and in playful locomotion by rolling – Ro A, Ro T. Together with *M-Sub* and *M-A,* they were often seen wrestling.

Table 1.5. The mean [median (min-max)] of behavioral element ***rate*** (behavior/ min.) of individual grey seals of different sex and age during the social PI (Sub-adult males – M Sub (n = 103), Sub-adult females – F Sub (n = 23), adult males – M A (n = 17), juveniles – Juv (n = 17), unknown – Unkn (n = 8)). Median test (MT) was used to estimate behavioral rate difference (when $p < 0,05$) between different sex and age classes. Behavioral abbreviations defined in Table 1.2. *N/A* – not analyzed due to low values.

Behavior	M Sub	F Sub	M A	Juv	Unkn	MT ($\chi 2$), p
Ap	0,18 [0 (0-2,5)]	0,07 [0 (0-0,41)]	0,07 [0 (0-0,49)]	0,31 [0,07 (0-1,67)]	0,11 [0,04 (0-0,6)]	$\chi 2 = 2,32$, $p = 0,68$
BHF	0,02 [0 (0-1)]	0 [0 (0-0,07)]	0,03 [0 (0-0,5)]	1,18 [0 (0-10)]	0	$\chi 2 = 2,37$, $p = 0,67$
B	0,02 [0 (0-0,47)]	0 [0 (0-0,06)]	0,17 [0 (0-2,78)]	0	0	$\chi 2 = 2,7$, $p = 0,61$
Mo	0,11 [0 (0-1,5)]	0,07 [0 (0-0,97)]	0,19 [0 (0-0,82)]	1,2 [0 (0-10)]	0,02 [0 (0-0,15)]	$\chi 2 = 4,3$, $p = 0,37$
Loc W	0,17 [0 (0-5)]	0,18 [0,12 (0-1)]	0,11 [0 (0-0,5)]	0,22 [0 (0-0,88)]	0,06 [0 (0-0,2)]	$\chi 2 = 8,49$, $p = 0,08$
W	**0,55 [0,57 (0-1,91)]**	**0,06 [0 (0-0,5)]**	**0,24 [0 (0-1)]**	**0,2 [0 (0-1,19)]**	**0,19 [0,17 (0-0,41)]**	**$\chi 2 = 25,39$, $p < 0,001$***
SI	0,24 [0 (0-2,5)]	0,11 [0,04 (0-0,48)]	0,56 [0 (0-7,5)]	0,49 [0,36 (0-1,67)]	0,06 [0 (0-0,2)]	$\chi 2 = 8,8$, $p = 0,07$
F	**0,04 [0 (0-2,5)]**	**0,08 [0 (0-0,7)]**	**0,67 [0 (0-5,56)]**	**0,64 [0 (0-10)]**	**0,15 [0,12 (0-0,4)]**	**$\chi 2 = 20,34$, $p < 0,001$***
Ro W	0,19 [0,03 (0-1,24)]	0,19 [0,18 (0-0,69)]	0,1 [0 (0-0,5)]	0,21 [0,11 (0-0,83)]	0,25 [0,19 (0-0,88)]	$\chi 2 = 6,32$, $p = 0,18$
Loc L	0,05 [0 (0-1)]	0,05 [0 (0-0,46)]	0,18 [0 (0-2,5)]	0,06 [0 (0-0,89)]	0,01 [0 (0-0,05)]	$\chi 2 = 2,68$, $p = 0,61$
A	0,19 [0 (0-3)]	0,12 [0,07 (0-0,41)]	0,25 [0 (0-2,68)]	0,23 [0,07 (0-0,83)]	0,04 [0 (0-0,16)]	$\chi 2 = 0,72$, $p = 0,95$

Behavior	M Sub	F Sub	M A	Juv	Unkn	MT (χ2), p
R	0,18 [0 (0-1,75)]	0,07 [0 (0-0,68)]	0,53 [0 (0-7,5)]	1,48 [0,3 (0-10)]	0,06 [0 (0-0,27)]	χ^2 = 5,72, p = 0,22
Mz	0,08 [0 (0-0,99)]	0,08 [0 (0-0,42)]	0,1 [0 (0-0,89)]	0,11 [0 (0-0,89)]	0,08 [0,07 (0-0,35)]	χ^2 = 4,63, p = 0,33
Ro B	0,15 [0 (0-2,5)]	0,16 [0,04 (0-0,6)]	0,08 [0 (0-0,49)]	0,12 [0 (0-0,83)]	0,19 [0,18 (0-0,49)]	χ^2 = 3,1, p = 0,54
L	0,18 [0 (0-5)]	0,13 [0,04 (0-0,68)]	0,06 [0 (0-0,42)]	0,14 [0 (0-1,22)]	0,19 [0,09 (0-0,65)]	χ^2 = 8,59, p = 0,07
NB	0,08 [0 (0-0,8)]	0,12 [0 (0-0,68)]	0,03 [0 (0-0,19)]	0,05 [0 (0-0,3)]	0,05 [0 (0-0,18)]	χ^2 = 1,25, p = 0,87
HS	0 [0 (0-0,5)]	0 [0 (0-0,06)]	0	0,1 [0 (0-0,89)]	0,13 [0 (0-1)]	χ^2 = 9,25, p = 0,06
P	0,05 [0 (0-0,74)]	0,05 [0 (0-0,39)]	0,04 [0 (0-0,5)]	0,04 [0 (0-0,49)]	0,02 [0 (0-0,09)]	χ^2 = 3,64, p = 0,46
Ch	0,11 [0 (0-1,5)]	0,07 [0 (0-0,73)]	0,36 [0 (0-2,78)]	0,09 [0 (0-0,83)]	0,04 [0,02 (0-0,09)]	χ^2 = 4,52, p = 0,34
Loc A	**0,06 [0 (0-0,68)]**	**0,18 [0 (0-1,5)]**	**0,25 [0 (0-2,78)]**	**0,15 [0 (0-0,83)]**	**0,41 [0,09 (0-2)]**	***χ2* = 12,65, p = 0,01***
OM+Voc	0,11 [0 (0-5)]	0,11 [0 (0-0,49)]	0,18 [0 (0-2,5)]	0,6 [0 (0-10)]	0,07 [0 (0-0,4)]	χ^2 = 6,16, p = 0,19
Ro T	**0,02 [0 (0-1)]**	**0**	**0**	**0,06 [0 (0-0,83)]**	**0**	***χ2* = 11,77, p = 0,02***
IM	0 [0 (0-0,09)]	0	0	0	0	*N/A*
Ro A	0,02 [0 (0-0,72)]	0 [0 (0-0,07)]	0,03 [0 (0-0,5)]	0,04 [0 (0-0,3)]	0	χ^2 = 2,59, p = 0,63
OMT	**0 [0 (0-0,05)]**	**0,05 [0 (0-0,68)]**	**0,05 [0 (0-0,89)]**	**0,07 [0 (0-0,82)]**	**0,02 [0 (0-0,16)]**	***χ2* = 9,79, p = 0,04***

Table 1.6. The mean [median (min-max)] of behavioral element ***proportion of the duration*** of individual grey seals of different sex and age during the social PI (Sub-adult males – M Sub (n = 34), Sub-adult females – F Sub (n = 7), adult males – M A (n = 4), juveniles – Juv (n = 7)). Median test (MT) was used to estimate difference in proportion of duration (significant differences, when $p < 0,05$, are bolded) between different sex and age classes. Behavioral abbreviations defined in Table 1.2. *N/A* – not analyzed due to low values.

Behavior	M Sub	F Sub	M A	Juv	MT ($\chi 2$), p
Ap	**0,02 [0,01 (0-0,1)]; n = 39**	**0,06 [0,06 (0,03-0,08)]; n = 2**	**0,01 [0,01 (0,01-0,02)]; n = 7**	**0,16 [0,07 (0,01-0,39)]; n = 6**	***χ2* = 15,55, p < 0,01**
BHF	n = 0	0,01 [0,01 (0,01-0,01)]; n = 1	0,01 [0,01 (0,01-0,01)]; n = 1	0,49 [0,49 (0,49-0,49)]; n = 2	*χ2* = 4, p = 0,55
B	0,1 [0,1 (0,1-0,1)]; n = 2	n = 0	n = 0	n = 0	*N/A*
Mo	0,04 [0,03 (0,01-0,16)]; n = 13	n = 0	0,02 [0,01 (0,01-0,04)]; n = 5	0,2 [0,29 (0,02-0,29)]; n = 3	*χ2* = 1,34, p = 0,93
Loc W	0,02 [0,02 (0-0,13)]; n = 18	0,01 [0,01 (0-0,02)]; n = 4	0 [0 (0-0,01)]; n = 3	0,04 [0,04 (0,01-0,07)]; n = 5	*χ2* = 6,23, p = 0,28
W	0,15 [0,07 (0-0,85)]; n = 145	0,05 [0,05 (0,05-0,05)]; n = 1	0,1 [0,07 (0,01-0,35)]; n = 25	0,09 [0,06 (0-0,34)]; n = 11	*χ2* = 1,61, p = 0,9
SI	0,03 [0,02 (0-0,22)]; n = 74	0,01 [0,01 (0-0,03)]; n = 7	n = 0	0,12 [0,07 (0,01-0,42)]; n = 12	*χ2* = 7,28, p = 0,2
F	0,01 [0,01 (0-0,04)]; n = 9	0,04 [0,04 (0,04-0,04)]; n = 1	n = 0	0,07 [0,01 (0,01-0,2)]; n = 3	*χ2* = 2,57, p = 0,77
Ro W	0,2 [0,11 (0,02-1)]; n = 44	0,19 [0,08 (0,03-0,9)]; n = 18	0,13 [0,05 (0,04-0,33)]; n = 7	0,29 [0,06 (0,02-0,68)]; n = 5	*χ2* = 5,38, p = 0,37
Loc L	0,02 [0,01 (0-0,06)]; n = 16	0,01 [0,01 (0,01-0,01)]; n = 1	n = 0	n = 0	*χ2* = 0,74, p = 0,98
A	**0,04 [0,02 (0-0,32)]; n = 54**	**0,02 [0,01 (0,01-0,05)]; n = 6**	**0,01 [0,01 (0-0,03)]; n = 4**	**0,07 [0,06 (0,04-0,14)]; n = 6**	***χ2* = 10,93, p = 0,05**

Behavior	M Sub	F Sub	M A	Juv	MT (χ2), p
R	0,06 [0,02 (0-0,91)]; n = 70	0,03 [0,02 (0,01-0,05)]; n = 5	0,04 [0,04 (0,04-0,04)]; n = 1	0,22 [0,06 (0,03-0,53)]; n = 7	$\chi 2$ = 5,6, p = 0,35
Mz	0,03 [0,02 (0,01-0,16)]; n = 11	0,03 [0,02 (0,01-0,07)]; n = 5	0,01 [0,01 (0,01-0,01)]; n = 2	0,09 [0,09 (0,01-0,16)]; n = 2	$\chi 2$ = 1,65, p = 0,9
Ro B	0,05 [0,02 (0-0,48)]; n = 35	0,13 [0,02 (0,01-0,48)]; n = 5	0,02 [0,01 (0,01-0,04)]; n = 5	0,04 [0,03 (0,02-0,07)]; n = 4	$\chi 2$ = 2,38, p = 0,79
L	0,02 [0,01 (0-0,1)]; n = 21	0 [0 (0-0,01)]; n = 3	0 [0 (0-0,01)]; n = 5	0,04 [0,04 (0-0,07)]; n = 2	$\chi 2$ = 4,02, p = 0,55
NB	0,03 [0,01 (0-0,2)]; n = 23	0,02 [0,01 (0,01-0,03)]; n = 6	0,01 [0,01 (0,01-0,02)]; n = 4	0,02 [0,02 (0,02-0,02)]; n = 1	$\chi 2$ = 2,06, p = 0,84
HS	n = 0	n = 0	n = 0	0,14 [0,14 (0,14-0,14)]; n = 1	*N/A*
P	0,03 [0,02 (0,01-0,07)]; n = 7	0,05 [0,02 (0,02-0,17)]; n = 7	0,05 [0,05 (0,01-0,09)]; n = 3	0,08 [0,08 (0,08-0,08)]; n = 2	$\chi 2$ = 3,72, p = 0,59
Ch	0,02 [0,02 (0-0,07)]; n = 9	0,02 [0,02 (0,01-0,03)]; n = 2	0 [0 (0-0)]; n = 1	0,07 [0,07 (0,07-0,07)]; n = 1	$\chi 2$ = 3,35, p = 0,65
Loc A	0,02 [0,01 (0-0,16)]; n = 16	0,05 [0,05 (0,05-0,05)]; n = 1	0,01 [0,01 (0,01-0,01)]; n = 1	0,05 [0,07 (0,01-0,07)]; n = 3	$\chi 2$ = 5,25, p = 0,39
OM+Voc	0,02 [0,02 (0-0,07)]; n = 7	0,02 [0,02 (0,01-0,03)]; n = 4	0,01 [0,01 (0,01-0,01)]; n = 2	0,11 [0,11 (0,01-0,2)]; n = 2	$\chi 2$ = 2,09, p = 0,84
Ro T	0,26 [0,26 (0,02-0,5)]; n = 2	n = 0	n = 0	0,07 [0,07 (0,07-0,07)]; n = 1	$\chi 2$ = 0,75, p = 0,98
IM	0,03 [0,03 (0,01-0,05)]; n = 2	n = 0	n = 0	n = 0	*N/A*
Ro A	0,01 [0,01 (0,01-0,01)]; n = 3	0,01 [0,01 (0,01-0,01)]; n = 2	n = 0	0,03 [0,03 (0,03-0,03)]; n = 1	$\chi 2$ = 6, p = 0,31
OMT	n = 0	n = 0	n = 0	n = 0	*N/A*

1.3.2. Scan Details

A total of 441 scans, covering 21 separate days of observations of the closest group at site A, were recorded over 4 to 9 h of observations per day. Of these scans, 304 included at least two individuals hauled out, while in 137 scans only one or no animals were observed hauled out at site A.

There was a median of 61,5 (7 to 211,5) individuals in the closest haul-out site A during observations and up to 720 individuals at a time. Of the total amount of observation time when grey seals were visible, 27% included social play. There was a median of 2,22% (0,99 to 3,53%) of grey seals per scan involved in PI. Up to nine dyadic interactions at a time were recorded.

Haul-out sites A and B were used most commonly because these areas dried out first; however, individuals in site A would usually move away and join the group at site B approximately an hour or two before low tide. Other haul-out sites were used only during the 2- to 3-h period before and after low tide as land became available (Fig. 1.5).

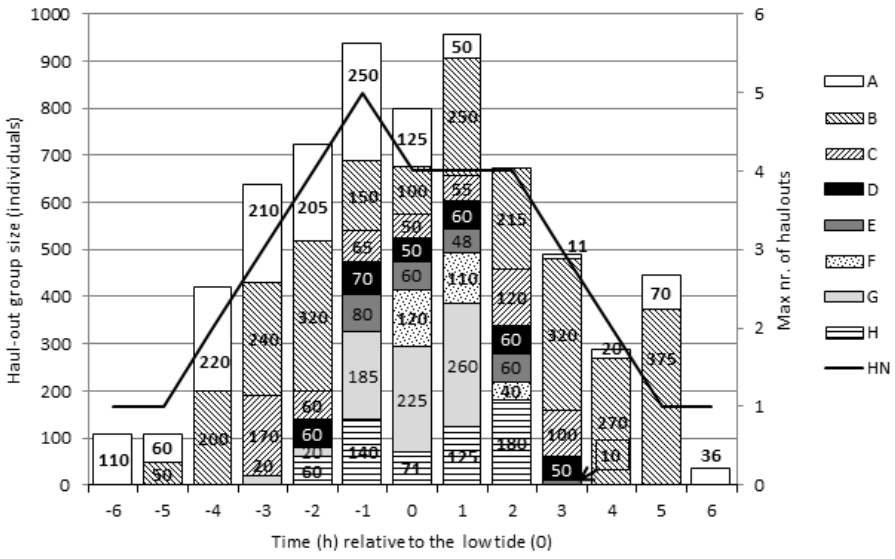


Fig. 1.5. The median number of individuals (presented as columns on the left y-axis) present in all haulouts (A-H) and a maximum number of haulouts (HN) in the study area (represented as the dark line scaled on the right y-axis) during the study period relative to tide time (h).

1.3.3. The Effect of Group Size on the Contact Rate, Number of Social Interactions, and Temporal Sequence of Play

There was a moderate positive relationship between both the group size and CR (Spearman, N = 304, R = 0,40, p < 0,001) and the group size and PI (N = 304, R = 0,46, p < 0,001). The closest haul-out site A consisted of a median of 36 (6 to 107) individuals when there were no PIs and 220 (106 to 312) individuals when there was at least one social PI (Mann-Whitney, N [CR = 0] = 225, N [CR > 0] = 79, U = 3,594, p < 0,001).

It was possible to record a group formation process in haul-out site A on 19 occasions. These occasions were identified from 201 scans taken at intervals of 15 min after at least two animals were seen hauled out on land. Groups lasted a median of 3 h (2,07 to 4,44). There was a positive relationship between group size and HFP (R = 0,5, p < 0,001). Groups had a median group size of 15 (4 to 115) individuals during the first hour after the beginning of group formation, which increased to a median of 250 (60 to 340) individuals after the third hour from the beginning of group formation (KW, H = 43,33, p < 0,001) (Fig. 1.6).

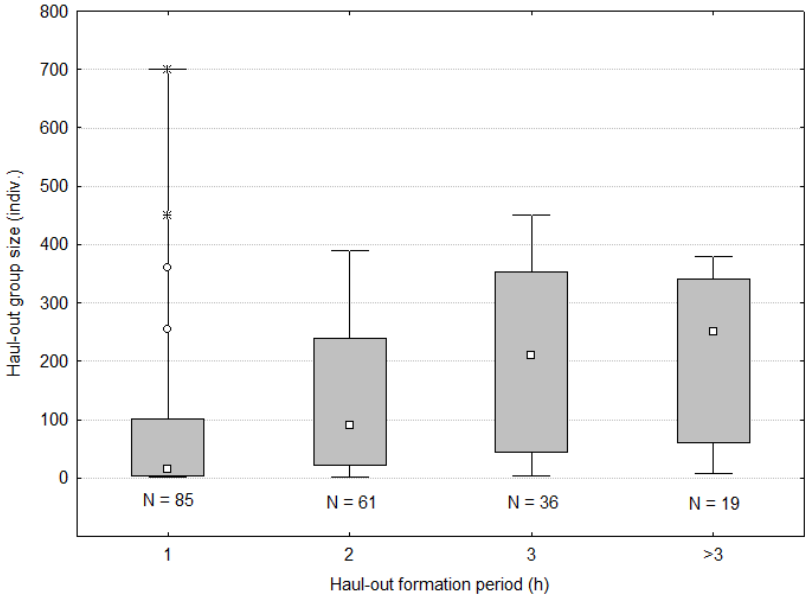


Fig. 1.6. Change in group size (i.e., number of individuals; H = 43,33, p < 0,0001) during haul-out formation period (HFP). Squares denote median values; boxes, Q_1-Q_2; whiskers; min-max; circles, outliers; and stars, extremes. Sample sizes are indicated below the graph.

There was a weak relationship between CR and HFP (Spearman, R = 0,17, p = 0,018) and between PI and HFP (R = 0,22, p < 0,01). Despite the low median values and no significant change in the CR (KW, H = 4,52, p = 0,21) during HFP (Fig. 1.7), there was an increase in the CR during the second and third hours that decreased slightly after the third hour of HFP. Similarly, the highest PI was observed during the third hour, and the lowest during the first hour; however, this change was significant (KW, H = 8,42, p = 0,038) (Fig. 1.7).

There was a change in the number of individuals and CR in the closest haulout depending on tidal state. The highest number of individuals was observed 3 h before the low tide, while there were almost no individuals from 1 h before the low tide to the second hour after the low tide (Fig. 1.8).

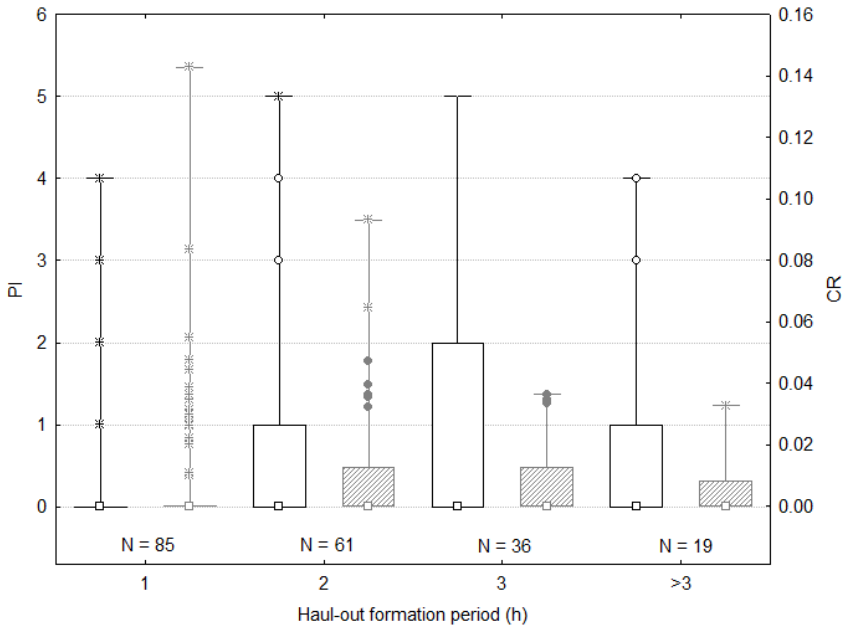


Fig. 1.7. Change in number of PI (white boxes) and CR (grey boxes) (H = 4,52, p = 0,21) during the period of HFP. Squares denote median values; boxes, Q_1-Q_2; whiskers, min-max; circles, outliers; and stars, extremes. Sample sizes are indicated.

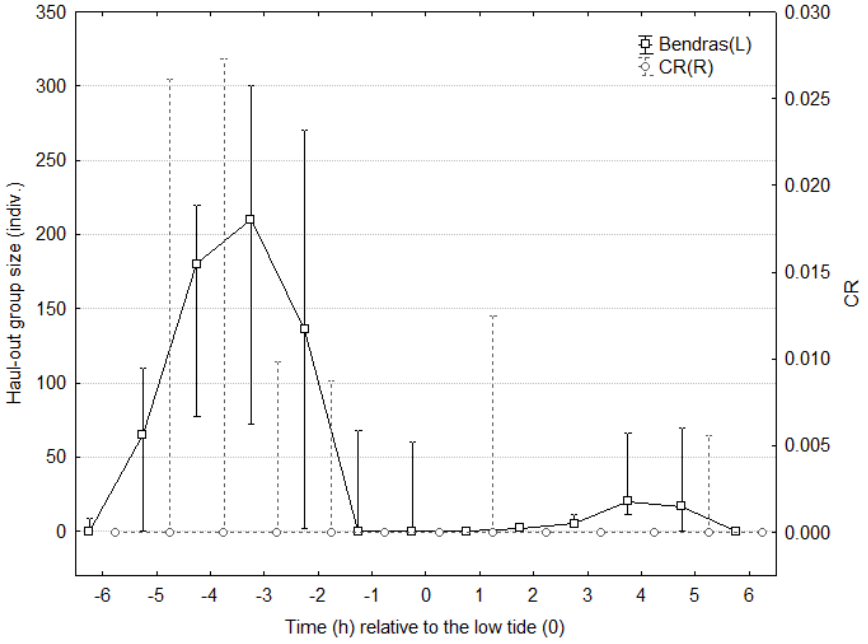


Fig. 1.8. Change in median group size (circles) and CR (squares) at low tide (0) (whiskers denote 25th to 75th percentiles); N = 441.

1.3.4. Group Composition and Social Play

The proportions of seals in each sex and age class in haul-out groups were unequal (Friedman's ANOVA, $\chi 2 = 830{,}93$, $p < 0{,}001$; Kendall's $W = 0{,}68$) (Fig. 1.9). Males (i.e., sub-adults and adults combined) predominated and composed 60% of the group. Adults made up more than 80% of those classified.

A Mann-Whitney test was performed to determine whether there was a significant difference in the proportion of different sex-age groups when there were no interactions vs at least one interaction in the closest haul-out site (Table 1.7). There was no difference in the proportion of males; however, the proportion of adult females decreased when interactions were recorded. The proportion of the population consisting of the demographic groups with the most playful individuals (i.e., juveniles, sub-adult males, and sub-adult females) was approximately 3% greater when play behavior was observed compared with periods of no interactions.

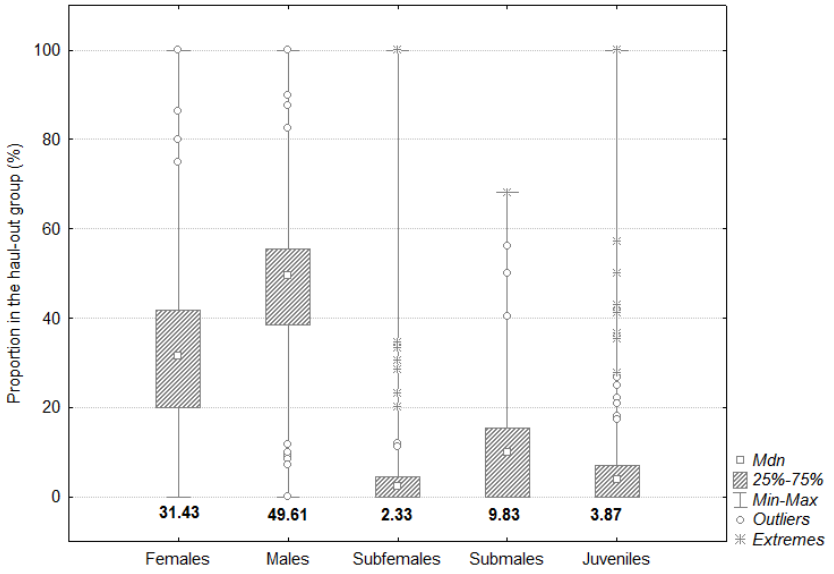


Fig. 1.9. Age and sex ratios of grey seals in the closest haul-out site A.

Table 1.7. a) proportion (%) of individuals of different age and sex in the closest haul-out expressed as median (Q_1-Q_3) when CR = 0 (N = 225) and CR > 0 (N = 79) and statistical indicators of differences between these groups (Mann-Whitney U test); b) Spearman rank correlations (R) between the proportion (%) of individuals of different ages and sexes in the closest group and CR. Significant differences are marked with asterisks (* < 0.05, ** < 0.01, ***< 0.001).

Proportion (%) of:	**When *CR* = 0 Q_2 (Q_1; Q_3)**	**When CR > 0 Q_2 (Q_1; Q_3)**	**U**	**p-level**	**Spearman rank correlation**
Adult females	33,33 (18,27; 50)	28 (23,23; 34,78)	7330	0,02*	R = -0,17 p < 0,01**
Adult males	48,94 (33,33; 56,14)	49,8 (46; 54,17)	7581	0,052	R = 0,11 p = 0,055
Sub-adult females	0 (0; 4)	3,57 (2,33; 6,57)	4820	< 0,001***	R = 0,36 p < 0,001***
Sub-adult males	8,77 (0; 16,22)	10,87 (8,57; 14,52)	6999	< 0,01**	R = 0,17 p < 0,01**
Juveniles	3,59 (0; 8)	4,93 (3,04; 6,49)	7045	< 0,01**	R = 0,16 p < 0,01**

CRs from groups with varying proportions of young individuals (juveniles and sub-adults altogether) were different (KW, H [N = 304] = 20,28, p < 0,001) (Fig. 1.10). Similar results were found regarding the relationship between

PI and the proportion of young individuals in a group (KW, H [N = 304] = 24,58, p < 0,001) (Fig. 1.11). The highest CRs were observed in groups in which young individuals comprised approximately 33 to 66% of the haul-out group, and the group consisted of approximately 101 to 200 individuals (Fig. 1.10); whereas the highest number of PIs was recorded when group size was between 201 and 300 individuals and consisted of 0 to 33% young individuals (Fig. 1.11).

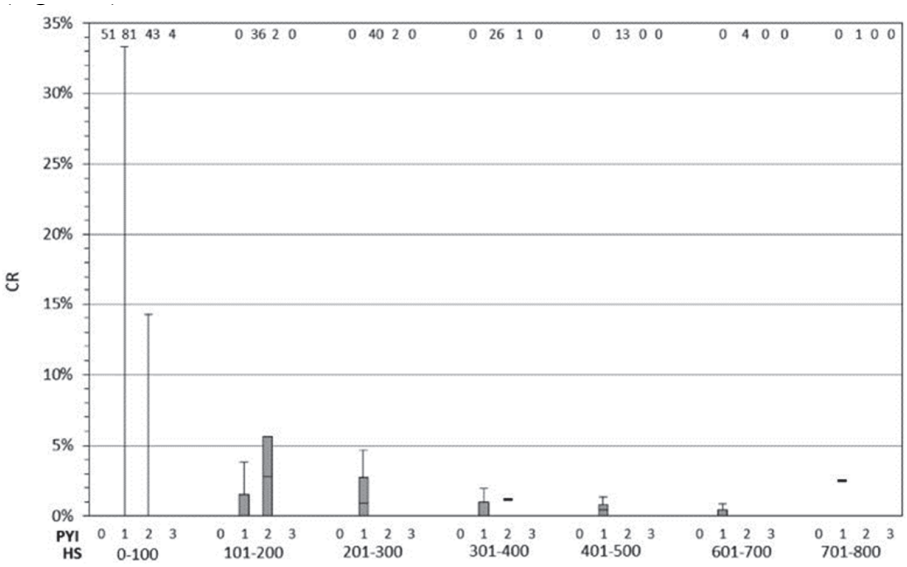


Fig. 1.10. CRs in different haul-out group sizes (HS, N = 304). The numbers in the PYI row beneath the chart indicate different proportions (%) of young individuals (PYI) in the closest group of grey seals: 0, 0% (N = 51); 1, 0 to 33% (N = 201); 2, 33 to 66% (N = 48); and 3, 66 to 100% (N = 4). Whiskers denote maximum values; middle lines, median values; and upper and lower columns, upper and lower quartiles.

The increase in CR during the second hour of HFP might be explained by an increase in the proportion of young individuals (juveniles and sub-adults altogether) (Spearman, R = 0,31, p < 0,001). There was a median of three individuals (0 to 16) during the first hour of HFP, which comprised 14,06% (0 to 27,08%) of the group size. During the second hour of HFP, a median of 23 (2 to 36) individuals were recorded, which comprised 16,67% (11,25 to 24%) of the group size in the closest haul-out site, and this change in proportion was significant (KW, H = 12,09, p < 0,01).

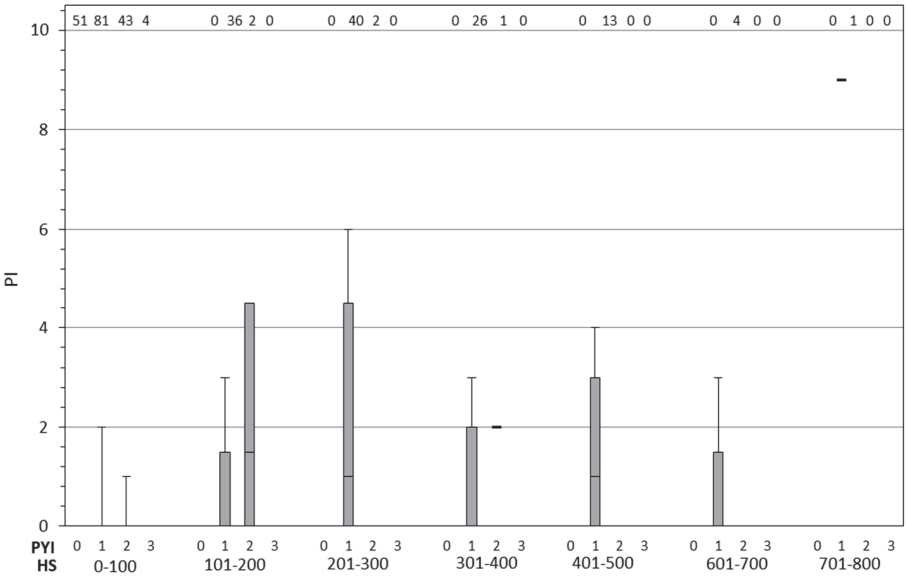


Fig. 1.11. Number of PIs on different haul-out group size (HS, N = 304). The numbers in the PYI row beneath the chart indicate different proportions (%) of young individuals (PYI) in the closest group of grey seals: 0, 0% (N = 51); 1, 0 to 33% (N = 201); 2, 33 to 66% (N = 48); and 3, 66 to 100% (N = 4). Whiskers denote maximum values; middle lines, median values; and upper and lower columns, upper and lower quartiles.

The highest correlation was found between subfemales and HFP (Spearman, R = 0,4, p < 0,001). On most occasions, there were no sub-adult females during the first hour, and their numbers increased to a median of 7 (2 to 17) individuals or 4,04% (2,32 to 7,21%) during the second hour (KW, H = 25,4, p < 0,001). Submales comprised the highest proportion of young individuals in the group and increased from a median of 2 (0 to 10) individuals or 6,06% (0 to 14,51%) during the first hour of HFP to a median of 31 (6 to 59) individuals or 12,40% (10 to 16,77%) after the third hour (KW, H = 12,23, p < 0,01) (Fig. 1.12). The relationship between submales and CR was weaker (R = 0,29, p < 0,001). The relationship between the proportion of juveniles and HFP was weakest among young (submale, subfemale, and juvenile altogether) individuals (R = 0,25, p < 0,001). There was a small difference in the proportions of juveniles during HFP (KW, H = 8,36, p = 0,039) (Fig. 1.12): juveniles increased from a median of 0 (0 to 5) individuals or 0% (0 to 5,43%) during the first hour of HFP to a median of 13 (2 to 14) individuals or 4,38% (3,06 to 5,31%) after the third hour; proportions remained stable between the second and fourth hour (Fig. 1.12).

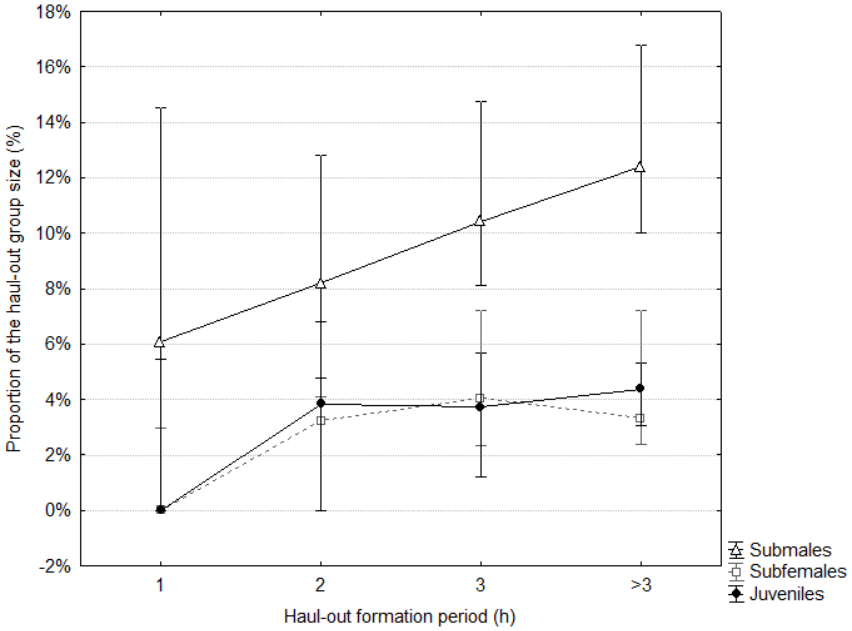


Fig. 1.12. The change in proportion of young individuals – submales (open squares), subfemales (grey squares), and juveniles (black circles) – during the HFP. Squares/ circles denote medians, and lines indicate the Q_1-Q_3 spread.

The proportion of adult grey seals decreased (Spearman, R = -0,31, p < 0,001) throughout the HFP, whereas the number of individuals in the group increased (R = 0,52, p < 0,001). There was no strong correlation between HFP and either the proportion of adult females or adult males separately. The median proportion of adult females remained around 33% (21,43 to 50%) during HFP (KW, H = 3,32, p = 0,34). The proportion of adults that were male did not exhibit any change during HFP (KW, H = 1,01, p = 0,8). However, the number of both adult females and males increased in the group during HFP (males: from a median of 5 [1 to 60] to 122 [39 to 140] [KW, H = 40,74, p < 0,001]; females: from a median of 4 [2 to 22] to 66 [12 to 119] [KW, H = 39,45, p < 0,001]).

1.3.5. Factors affecting contact rate

The best model was described by 3 significant variables (AIC = 485,25, Likelihood Ratio Chi-Square = 105,99, df = 3, p < 0,001) (Table 1.8). The number of PIs increased with number of individuals in the haul out group and throughout the observation period, but decreased slightly with time relative to the low tide, i.e. with dry land available.

Table 1.8. NBGLM statistics for play interactions (number of PIs). *B* ± *SE* – model coefficient with standard error, *Lower/ Upper 95% Wald CI* – lower and upper 95 % confidence intervals for Wald test, *Wald* χ^2 – Wald chi square statistics, *df* – degrees of freedom. Model is fixed when *PI* = 1.

Parameter	***B* ± *SE***	**Lower 95 % Wald *CI***	**Upper 95 % Wald *CI***	**Wald χ^2**	**df**	**p**
Intercept	-5572,23 ± 1434,24	- 8383,29	-2761,17	15,09	1	< 0,001
Haul-out size	0,005 ± 0,001	0,004	0,007	53,26	1	< 0,001
Time relative to Low Tide	-0,17 ± 0,04	- 0,25	- 0,1	20,05	1	< 0,001
Date	4,14E-07 ± 1,07E-07	2,05E-7	6,22E-7	15,09	1	< 0,001

1.4. DISCUSSION

1.4.1. Characteristics of grey seal social play

In this study detailed characteristics of grey seal social play were described for the first time and indicate that it might be used to prepare for future breeding seasons. Sub-adult males were the most active players, even though they were not the most abundant sex and age group in the haul-out area. Young grey seal males, like other colonial pinniped species (i.e., fur seals (Arnold and Trillmich, 1985; Harcourt, 2010), Steller sea lions (Gentry, 1974), and elephant seals (Reiter et al., 1978)), as well as well studied laboratory rats (Panksepp and Beatty, 1980; Pellis and Pellis, 1990), were more social and playful than females, most likely because many of the physical and psychological aspects of play help prepare them for future intraspecific combat, whereas females mature early (Pomeroy et al., 1999) and interact less physically (Boness and James, 1979; Burghardt, 2005).

In common with many other polygynous colonial species (Arnold and Trillmich, 1985; Gentry, 1974; Harcourt, 2010; Reiter et al., 1978), play fighting or wrestling is one of the main elements observed during social play for sub-adult male grey seals. More importantly, social play closely resembles male behavior during the breeding season (Bishop et al., 2015b; Twiss, 1991). All main behavioral elements, such as wrestling, lunging, flippering, biting hind flippers, biting, alert (social investigation in this case) and open mouth vocalization were present during social play. However, all elements differed in their intensity and consequences. Indeed, social investigation resembled alert or looking to another male (Twiss, 1991) behavior which involved intensive staring at each other with heads kept at different heights. Both these behaviors initiated the subsequent behavior, which would often be a play fight as in this study or real fight during the breeding season (Twiss, 1991). There were no statistical differences in social investigation between different groups, indicating that the behavior is universal among different age and sex groups. Play fight behavior or wrestling was identical in appearance to adult male fights during the breeding season reported by Twiss (1991), the only difference was that agonistic activities, such as biting the back of the neck or the back itself was not violent, as biting was somewhat restricted to mouthing, where mouth was not fully closed and no shaking was observed. A significant difference between different age and sex individuals while performing this behavior was observed. Sub-adult males had the highest

behavioral rate as well as proportion during the PI while sub-adult females had the lowest contact rate which indicates the importance for practicing this behavior in males. Adult males and juveniles had twice as low wrestling rate. One of the explanations could be related to different motivation for social play in adult seals as it is well studied in other mammals (Fagen, 1981; Pellis and Pellis, 1998, 1990). There is a specific developmental window when key brain structures responsible for motor functions and habit formation, like medial prefrontal cortex, are developing and motivation for social play is the highest so that young individuals could successfully practice skills important for adaptation to the present environment and future behavior (Ikemoto and Panksepp, 1992; Panksepp and Beatty, 1980). Young sub-adult males seem to be in the motivational peak for social play even though they should be physically matured. On the other hand, locomotion on land requires energetic resources as movements are restricted compared to movements in the water (Garrett and Fish, 2015; Tennett et al., 2018) and better coordination to save energy. This would explain the lower wrestling rate in juveniles who have comparably higher metabolic rate (Worthy, 1987; Worthy and Lavigne, 1987) and lower energetic resources. Higher energetic on-land demands could also explain higher rates of interruptive behavior, such as flippering, open mouth threat in juveniles and adult males, and locomotion away in sub-adult females and adult males that indicates lack of willingness to participate in the contact or intention for a break to get rest. This "lack of willingness to participate" corresponds with the observations of rat social play in which defensive behavior is more frequent in very young pups, who just start to socially interact, and those young rats that come closer to maturity (Pellis and Pellis, 1990). According to Bekoff and Allen (1997), agonistic interactions usually terminate the play and unfair individuals are pushed away from the group. Although, it was not observed in detail in this study, since the focus was on the playing pairs, it was observed on several occasions when agonistic response by adult males pushed away playful individuals and PI ceased. Similarly, approach behavior took significantly more time during the PI in sub-adult females and adult males showing that they reinitiated social play after a break. Most of rolling towards another individual took place in juveniles and sub-adult males. Rolling was observed as a behavior of victory and calming down by a winner male after the intensive fight during the breeding season (Twiss, 1991). However, rolling was showed only during prolonged PIs, mostly to initiate the play again or keep it going, as it would encourage a partner to

approach and pin or mount individual performing the rolling. Here rolling serves not as a victory, but more as a more comfortable movement during PIs that maintains the play and also puts an animal into a vulnerable position, because it exposes the belly and the neck, and might have limited amount of time to react to a potential danger, thus an animal must be confident to do that in front of the partner and familiar with play rules.

Sexual social play pattern – mounting, neck biting, imitation of mating, was observed mostly amongst sub-adult females and males, and resembles the behavior during the breeding season showing the importance of practicing sexual behavior (Twiss, 1991). However, differently from breeding behavior, a copulation was never achieved, as sub-adult females would always terminate it by flippering and moving away from a partner, although a playful interaction could be reinitiated soon after. There is little knowledge of how and when females are impregnated for the first time. Imitation of mating could be an attempt to copulate as the position of two animals, the sex of the players and the thrusting behavior of a male on a top would indicate as this was the case. In wild chimpanzees (*Pan troglodytes schweinfurthii*) the amount of play was correlated with first observed mating attempt, therefore increasing the chances for reproductive success (Heintz et al., 2017). The lack of willingness from female side might indicate that they are not in an ovulation phase, similar to observations in the wild during the breeding season (Twiss, 1991). Ovulation usually starts at the end of lactation during the breeding season (Pomeroy et al., 2001). Although, as mentioned before, no clear indication when females that never gave birth start to ovulate, it must take place somewhere at the end of September – two months later than the observation period. Thus social play might be a prelude to the breeding behavior for sub-adult females.

Playing individuals (mostly sub-adults and juveniles) were always at the periphery of the group, usually at the edge of the water and were observed by surrounding individuals of various age most of the time. The middle of the colony was occupied by already resting adults thus no interactions were visible in that area. Any interactions performed in a middle of the colony would have caused disturbance and aggression from resting individuals therefore terminating play behavior or making playing individuals move to the more open edges of the colony. During the breeding season males compete for access to females, not dominant, usually young males, roam on the edge of the water and usually mate with females there (Boness et al., 2014; Boness and James, 1979; Lidgard et al., 2005). Thus, in general the social play-fight

pattern during social play could imitate a fight for haul-out space – "territory" – or just a preparation for the breeding season as it was seen with weaned southern elephant seals (Reiter et al., 1978). It was shown with cheetahs that the amount of contact play is closely related with the number of contacts during adult behavior (Caro, 1995; Palagi et al., 2015). Infants of wild chimpanzees (*Pan troglodytes schweinfurthii*), who engaged in more social play would achieve motor and social milestones at younger ages, the amount of social play in this species also correlated with earlier ages of spatial independence from the mother and a first grooming of a non-maternal kin (Heintz et al., 2017). However, the structure and organization of social group of cheetahs and chimpanzees is different than that of grey seals, thus it might be that there is a different and more acceptable explanation. According to many authors (Pellis et al., 2010; Vanderschuren and Trezza, 2013), social play facilitates the development of social, cognitive, emotional, and motor skills, in particular the ability to use these capacities flexibly in a changeable and unpredictable environment (Pellis and Pellis, 2009; Špinka et al., 2001; Vanderschuren and Trezza, 2014). Therefore, the impact of social play on grey seal cognitive and motor development should be investigated more in the future research.

Even though not reported in result section, one interaction would usually serve as a trigger for other animals to engage in play, especially juveniles, and on some occasions five sequential interactions could be observed. Once two animals would start playing, smaller or equal size individuals began to play beside them. Sometimes, three or four pairs were seen playing beside each other (author, pers. obs.). This contagion effect first time reported in grey seals was seen with weaned southern elephant seal pups (Reiter et al., 1978), as well as in other mammals, such as laboratory rats (Held and Špinka, 2011; Reimert et al., 2013) and dogs (Bekoff, 2015; Palagi et al., 2015). This indicates how important this behavior is for young individuals and the motivation to play at this age is high, as they seem to be sensitized to social play behavior.

1.4.2. Water vs. land play

The majority of the play behavior recorded was performed in shallow waters. However, there was an unknown proportion of PIs performed in the water, where most observations ceased due to lack of ability to observe them. During this research we were restricted to observations of shallows and land. Behavioral elements, such as wrestling, mounting, neck-biting, rolling together and on the back, required an intimate connection between grey seal

individuals, while play in the water allows more opportunities to escape and more freedom to move. Grey seals might need to gain experience to play on land, become familiar with a partner or social play on land itself. This could explain a lower amount of juveniles participating in PIs on land. Social interactions of captive weaned pups begin in the water as play chase and at least a month of spending time together is necessary for them to start close contact play on land (author, pers. obs.).

Also differently from water, play on land requires more energetic recourses, because movements of true seals are more restricted on land (Garrett and Fish, 2015; Tennett et al., 2018). This might have caused higher frequency of resting and alert behaviors – "pauses", during social play for young individuals, especially for juveniles, because of their higher metabolic rate in general and lower energetic resources (Worthy, 1987).

1.4.3. Group Size and Social Play

These results support the hypothesis that haul-out group size has a positive effect on grey seal socialization. As it was shown by generalized linear mixed model, play behavior is positively related to the number of individuals in a group and was most commonly performed when there were more individuals in a haulout than in a haulout with no interactions (median group sizes 220 vs 70 individuals).

According to the hypothesis, based on Surplus theory of play (Burghardt, 2005), the number of PIs and the contact rate should increase with the number of individuals in the haul-out group. However, this relationship was not consistently observed. The highest number of interactions and the highest contact rate were observed when there were between 100 and 200 individuals in the closest haul-out group. This group size was reached during the second and third hours of HFP. As expected, there was a stronger relationship between group size and PIs, because there are more potential players in a bigger group. However, contact rate reflects the actual proportion of playing individuals and provides more information regarding how PIs increase with group size.

The inconsistent increase in contact rate with group size may be explained by tidal state and group structure and formation, particularly with respect to differences in the organization of adjacent haul-out sites in the research area and the use of the closest haul-out space. This was also confirmed by generalized linear model where number of contacts was negatively related with the haul out formation period. Haul-out group formation was closely

related to the water level; thus, group size changed according to tidal state. Seals would start to haul out immediately after the sand bank appeared above the water. However, instead of constantly increasing towards the low tide, the number of individuals in haul-out site A stopped increasing and started to decrease approximately 3 h before low tide or after the third hour from the start of group formation. This phenomenon occurred because animals tended to leave the closest site A and join other haul-out sites towards low tide, most likely to avoid being left too far away from the water as the tide ebbed. Indeed, the number of haul-out sites increased as the low tide approached because the receding water would disclose more sand banks, and they would start to decrease just after low tide. The first groups of grey seals to leave the closest site A would usually swim away. The remaining group of grey seals would join another close site B, traveling all the way (approximately 100 m or more at a time) on land. They would usually return during the first or second hour after the low tide, and the formation of the haul-out group A would start again.

The low CR after low tide might have been related to lower numbers of individuals in the group compared with the group size just after the high tide. It might also have been due to the activity of animals because grey seals spent most of their time on the haul-out site resting (author, pers. obs.). Thus, the second explanation for the variation between group size and CR might simply be that grey seals tended to enter a resting state after they hauled out. Even those animals that played on the edge of haul-out sites usually did it temporarily and later hauled out completely. This corresponds with results observed in harbor seals by Wilson (1974) in which play behavior eventually resulted in two playing individuals hauling out on land next to the remaining group and entering a resting state. As a result, there still would be similar numbers of individuals playing since some individuals stop playing and enter the resting state while new ones come on land and begin to play; however, the contact rate stopped increasing at a certain period or remained stable, while the group size continued to grow possibly due to the increasing proportional ratio of resting vs. playing individuals.

Data from separate observations from the surrounding haul-out sites around site A demonstrated that grey seals tended to distribute themselves into separate haulouts (up to five at a time) of 100 (50 to 250) individuals when space was available. This tendency to scatter instead of forming one large group requires further observations. However, the median number of individuals in a group is very close to the overall group size when the highest

CR was seen (100 to 200 individuals). Groups of this size might ensure that all individuals can move within or leave the haul-out site without a delay and allow animals to socialize and participate in play behavior.

1.4.4. Group Composition and Social Play

During haul-out group formation, not only group size but also group structure was changing. Therefore, group structure was another factor affecting grey seal social play behavior. The results suggest that CR and play behavior have a positive relationship with the proportion of young individuals in a group, although this relationship is weak. As was previously mentioned, this might be related to the fact that on most occasions, the active players – sub-adults and juveniles – would haul out at the end of a bout of play, similar to grey seal observations by Wilson (1974). Thus, there was an increase in the proportion of sub-adult males; however, the number of play behaviors remained the same or started to decrease.

Juveniles and sub-adult females, which are very conspicuous and come only during the second hour of group formation, might be attracted by already playing individuals on shore (e.g., sub-adult males) because the behavior indicates a safe environment for them to haul out. This may be particularly applicable to sub-adult females, who exhibited the strongest positive correlation with CR.

The number of adult individuals might have an indirect effect on the number of young individuals. Adults not only formed the core of the group and comprised 80% of the group size most of the time (Fig. 1.12), they were also the first to haul out on land (Survilienė, pers. obs.). However, adult males were rarely seen playing, and no interactions were seen among adult females. Adult females usually spent time resting, occasionally displaying agonistic behavior towards each other over space or checking the environment. It is known that adults are not very playful in general (Burghardt, 2005; Fagen, 1981). Although adults had either a negative relationship (adult females) or no relationship (adult males) with CR and had no direct effect on the CR, given that they formed the majority of the group, they might indirectly serve as an attractive factor for the main social play interactants (i.e., young grey seals).

Adult and sub-adult males together comprised approximately 60% of the group most of the time. Sexual segregation of grey seals has been observed in other areas throughout the UK. Leeney et al. (2010) reported that 80% of grey seals at haul-out sites in the Celtic Sea were male; however, they noted

that this ratio might be due to the different locations of females such as in caves or regions where prey is more abundant. Similar results were found by Sayer et al. (2012). A high male ratio was also observed on sand banks at Tentsmuir during the molting season (Pomeroy, pers. obs.). Conversely, Kiely et al. (2000) found a considerably higher proportion of females hauled out on the southeastern coast of Ireland. It is often stated that segregation of grey seals is due to inter-sexual competition and niche separation (Breed et al., 2006; Breed, 2008), which lead to different diet, diving, and spatial patterns (Beck et al., 2003a, 2003b, 2007; Breed et al., 2009). These hypotheses often disregard social behavior as a potential factor in the formation of aggregations. However, social factors might indeed cause sexual segregation on land, at least for young grey seals, because sub-adult male grey seals dominate social play behavior. Ruckstuhl and Neuhaus (2002) in their social preference hypothesis, based on studies of ungulates, other poligynous species structurally similar to grey seals, proposed that sexual segregation is caused by social affinities among males. They stated that even though similarities in activity budgets and nutritional requirements should be the main forces that lead to segregation of individuals of a particular sex, social interactions should facilitate sexual segregation at least among young male individuals. Additionally, in his later discussion Ruckstuhl (2007) discussed socio-preference hypothesis in more detail and proposed that there must be an innate preference for males to interact and to group with other males and for females to be with other females, and that these preferences alone lead to social segregation. One of the main reasons for male based than mixed groups is that this is where they learn and develop fighting skills and establish a dominance hierarchy (Michelena et al., 2005, 2004; Pérez-Barbería et al., 2005; Whiteside et al., 2017). Several authors argue that sexual differences in the social repertoire and motivation to interact with same-sex peers early in life as well as indifferences to, or avoidance of the opposite sex may have long-lasting consequences for social dynamics and grouping outside the mating season (Bon and Campan, 1996; Calhim et al., 2006). Social play might be one of the reasons for grey seals to form male-oriented haul-out aggregations in which sub-adult males have a greater likelihood to meet potential playmates (and future rivals). Social play offers a chance to practice fighting skills and might be one of the indicators of a safe environment for juveniles and sub-adult females to haul out.

The primary goal for grey seals to haul out outside the breeding season is to molt, reduce energetic costs during rest and possibly digest (Brasseur

et al., 1996; Davis, 2019; Watts, 1996). The group segregation during the haul outs increases vigilance for detection of potential threats (Da Silva and Terhune, 1988; Terhunet and Brilliant, 1996; Watts, 1996). Therefore the size itself appears to be an attraction force for young individuals possibly as it guarantees safety. Research on seal interactions with fishing gear in the Baltic Sea demonstrated that adult male grey seals raiding fishing nets are followed by young grey seals (Königson et al., 2013, 2007), thus segregations of same sex individuals might take place under water as well. As mentioned before, grey seals have strong site fidelity to their specific haul-out sites outside the breeding season (Karlsson et al., 2005; McConnell et al., 1999; Sjöberg and Ball, 2000). Thus, there is the opportunity for individuals to meet the same partners over a prolonged period of time, practice their social skills with similar individuals and form associations in a unsubstantiated term.

1.4.5. Future perspective of behavioral analyses using modern technologies

New technologies allow us to track mammals in real time and space. Spatial telemetry is widely used to monitor the location, movements and speed of marine mammals, including grey seals (Breed et al., 2009; Carter et al., 2017; McConnell et al., 1999; Sjöberg et al., 1995, 1999; Sjöberg and Ball, 2000). Tri-axial accelerometer tags provide quantitative data on body movement that can be used to characterize behaviour and understand species ecology in ways that would otherwise be impossible (Fehlmann et al., 2017; Lush et al., 2016). Efforts of joining telemetry trackers, equipped with a tri-axial acceleration sensor, allowed to correctly estimate around 90 % of the terrestrial behavior of red deer (*Cervus elaphus*) (Löttker et al., 2009), domestic goats (*Capra hircus*) (Moreau et al., 2009) and polar bears (*Ursus maritimus*) (Pagano et al., 2017).

Classifying behaviour with animal-borne accelerometers has recently become a popular tool for remotely observing behavioural states in a variety of pinniped species (Ladds et al., 2017; Viviant et al., 2010), but most of this work has focused on classifying behaviour at sea often quantifying behavioural trade-offs associated with foraging and diving. Several attempts were made to classify the behavior of adult lactating females during the breeding season using accelerometers (Shuert et al., 2020, 2018), thus accelerometers are a promising tool for grey seal behavioral classification.

More efficient remote video recordings of animal behavior enable scientists to gather more data for behavioral analysis, however usually such

recordings are performed during a breeding season in combination with in field observations (Bishop et al., 2015a; Robinson et al., 2015; Shuert et al., 2020; Sean D. Twiss et al., 2012). Otherwise, remote video observations are mostly used to estimate habitat use and distribution outside the breeding season (Clermont Edrén et al., 2005; Leeney et al., 2010). An alternative, but expensive and rarely used method is the use of animal mounted underwater cameras (Aoki et al., 2013; Hooker et al., 2015; Sato et al., 2003). The detailed behavioral observations of underwater social behaviors of marine mammals, like grey seals, during the non-breeding season would be possible only in captivity, with a hope that remote animal mounted cameras will be more often used in the wild.

Therefore combining a modified GPS tracker with a long lasting battery, that allows a good amount of data to be collected and a possibility to track an animal in real time, together with a tri-axial accelerometer and real time or remote observations of actual behaviors can provide a unique set of behavioral data during a socialization period.

1.5. CONCLUSIONS

Analysis of social play during non-breeding season revealed that:

1. The majority of interactions were performed by sub-adult males (61,76 %), followed by sub-adult females (13,53 %). Main interactions took place between sub-adult males (42,35 %) and between sub-adult females and sub-adult males (21,18 %).
2. Majority of grey seal behavioral repertoire consisted of play fight elements, mostly wrestling (14 %), which was most frequently expressed during sub-adult male social play interactions. Therefore social play may serve as a preparation for the breeding season.
3. Contact rate and number of social play interactions correlated with group size predominated (50 %) by adult males. Highest contact rate was observed when group consisted of 33-66 % of young individuals with the size of 100-200 animals hauled out.
4. Only group size, availability of haul out space and time closer to the breeding season had a significant positive effect on the number of play interactions.
5. Contact rate of social play had a weak positive correlation with haul out formation time. It was the highest during the second and the third hours of a haul-out group formation.

2. STUDY OF STEROID HORMONES IN RELATION TO BEHAVIOR IN SUCKLING AND WEANED GREY SEAL PUPS

2.1. LITERATURE REVIEW

2.1.1. Early development of grey seals

In mammal species the early developmental period usually includes a huge range of different periods from the formation of the embryo to animal's maturity (Lindström, 1999) and is critical for the formation of physiological, humoral processes and social behavior (Schulz et al., 2009; Sisk et al., 2003). In mammals the early postnatal period is often associated with a suckling period of various extents that meets high energetic demands of a newborn offspring. Grey seals are pagophilic pinnipeds, meaning that they are adapted to breed on a pack-ice, where they have a rapid access to deep waters, however this unstable drifting and changing substrate can lead to a separation between a mother and a pup. As a consequence of breeding on the unstable pack-ice, pagophilic seals have the shortest lactation period in pinnipeds (4–30 days), followed by a substantial period of a post-weaning fast (German Riet Sapriza, 2020). The early postnatal period of grey seals – the nursing (17-21 days) and fasting periods (5-40 days), a time during which they learn to feed themselves – will be discussed (Atkinson, 1997; Bennett et al., 2010; Kovacs and Lavigne, 1986a; Noren et al., 2008; Øritsland et al., 1985).

Grey seal females are capital breeders and give birth once a year to one pup within days of coming ashore (Mellish et al., 1999a; Pomeroy et al., 2000). They start breeding at 4-5 years of age, and continue to give birth annually for decades or more (Iverson et al., 1993; Pomeroy et al., 2000). The Baltic grey seal breeding season starts in late February, when the sea ice cover reaches its maximum coverage and thickness (Jüssi et al., 2008). The Western Atlantic grey seal population start their breeding season in January (Iverson et al., 1993), while the Eastern Atlantic – in late September (Pomeroy et al., 2000). Females become fertile at the end of lactation, approximately 3 days before weaning (Atkinson, 1997).

Depending on the breeding grey seal population and season, newborn male and female pups might have a different body mass at birth. In the West Atlantic population newborn grey seal male pups weight about 15,8 kg at birth,

while females – 14,8 kg (Bowen, 2016), while in the East Atlantic population individuals of both sexes weigh around 16 kg (Pomeroy et al., 1999). In the Baltic newborn grey seals are smaller and weigh about 12 kg. The weight between different sexes does not differ significantly (Jüssi et al., 2008).

Lactation lasts only 16-18 days, during which grey seal females do not feed, losing about 50-75 kg (25,8 % - 39 %) of their body mass (Iverson et al., 1993; Pomeroy et al., 1999). Females primarily utilize their blubber fat as the energy source, this way supporting both the metabolic processes of her body and the production of extremely fatty milk (Iverson et al., 1993; Iverson and Bowen, 1995). Meanwhile, the pup triples its weight, gaining an average of about 1,6 – 2,8 kg per day depending on the colony (pups in West Atlantic population are heavier at weaning) (Bennett et al., 2010; Bowen et al., 1992; Pomeroy et al., 1999).

Analysis of milk from grey seals has shown that the composition of milk changes during lactation. In the first several days, the fat content of milk is relatively low (~ 35 %), still very high compared to human or cow milk though, while water content is high (~ 50 %) (Baker, 1990). By the end of lactation, the situation reverses with fat content reaching up to ~ 54 % and the water dropping up to 31 %. The protein content slightly decreases from ~ 10% to ~ 9 % by weight of milk, but in general remains constant (Baker, 1990). The overall weight transfer efficiency (100 * pup weight gain / maternal weight loss) is significantly lower at the beginning of lactation (~ 47 %) than at the end (~ 63 %) (Iverson et al., 1993; Mellish et al., 1999a). Therefore, separation of pups from their mothers prematurely are very dangerous and can have negative effect on survival, as pups do not receive most of the energetic resources during late lactation.

The weaning process of grey seal pups is abrupt. Females leave to the sea or start mating with males (Atkinson, 1997). Male pups after weaning in the North Atlantic weigh on average about 40 – 56,2 kg, and females - 36 – 51,6 kg (Bowen, 2016; Hall et al., 2002; Pomeroy et al., 1999). Whereas, pup weight at weaning in the Baltic Sea range from 48,3 (± 8,1 kg) for those born on ice to 37,4 (± 7,8 kg) for those born on land (Jüssi et al., 2008). Therefore, the final body weight of the young varies greatly depending on the breeding location, mothers body size, experience, age, water availability and colony density, as discussed below.

The reproductive success of a female is measured by pup survival rates. First-year survival of a grey seal pup increases with a body mass and energy

reserves at weaning, as in many other pinniped species (Anderson et al., 1979; Hall et al., 2002, 2001). Larger pups with larger blubber fat reserves at weaning have a higher probability of survival (Anderson et al., 1979; Hall et al., 2001; Muelbert et al., 2003). The quality of lactation process and the energetic input of a mother guarantees better survival rate of her pup and depends on her body mass, age and experience as well as environmental factors, such as colony density and water availability (Redman et al., 2001). Larger mothers give birth and wean larger pups with higher survival rates (Bowen et al., 2015; Hall et al., 2001). In addition, females with lower body mass during lactation tend to wean their pups earlier or have smaller pups than larger dams due to unequal energy investment (Iverson et al., 1993; Mellish et al., 1999a; Pomeroy et al., 1999). The age of a female is an another significant factor for pup's survival as it is related with female's experience, mammary gland performance and the body mass of the female itself (Bowen et al., 2006; Lang et al., 2011, 2009; Pomeroy et al., 1999). Younger females give birth to smaller pups or have a limited experience to feed them. For example, they might start giving birth at the end of the breeding season when male harassment is most pronounced (Boness et al., 2014; Bowen et al., 2006; Lang et al., 2009).

Energy investment can also depend on the pup's sex. In some populations male pups are larger than female pups both immediately after birth and after nursing (Bowen et al., 1992). They also spend more time feeding and require more attention, and thus energy, from their mothers (Anderson and Fedak, 1987; Kovacs and Lavigne, 1986b, 1986a). Therefore, both younger (due to low weight and/or lack of experience) and older females (due to reduced physiological condition) are thought to be unable to successfully feed male pups or they wean weaker ones (Bowen et al., 2006). This is especially true for male pups whose first-year survival probability is up to 30 % lower (Hall et al., 2001).

Weaned pups remain on shore/ice for up to 40 days during the post-weaning fast (Bennett et al., 2007). During this fasting period, specific physiological changes of diving capacity (e. g. increased blood volume, hematocrit, myoglobin concentration, muscle development, etc.) take place (Noren et al., 2005). At the same time pup faces significantly higher energy demands to maintain a constant body temperature, swim and find food (Øritsland et al., 1985). During this period, pups lose about 10 kg (~30% of their body mass), axillary girth decreases about 13 cm, and the blubber fat layer thins about 1,5 cm (Øritsland et al., 1985). In grey seals, as in many other true

seal pups, experiencing a negative energy balance during post-weaning fast, catabolism of dry body mass is much more intense than in terrestrial mammals (Øritsland et al., 1985; Stewart and Lavigne, 1980). Therefore, in pups with lower body mass, this high metabolic rate leads to a rapid weight loss and a lower probability of survival. In extreme cases, when pup blubber thickness drops below a critical level, they are at risk of fatal hypothermia, especially when in the water (Øritsland et al., 1985).

Although during the post-weaning fast, 90% of energy requirements are met by fat catabolism of blubber fat reserves (Mellish et al., 1999b; Nordøy et al., 1990; Reilly, 1991), protein resources are very important for the survival of the young (Bennett et al., 2007). During the post-weaning fast, pups body use body's reserves for both, metabolic fuel and tissue production and functioning. Blubber fat layer is very important for true seals living in cold climatic zones, because it functions not only as an energy reserve, but also as an insulating agent, preventing the body from losing heat and freezing, especially when pup enters the sea (Jenssen et al., 2010; Lydersen and Kovacs, 1999; Øritsland et al., 1985). Proteins are low in energy but play an important role as the main structural and functional component of tissues. Even with sufficient adipose tissue resources some proteins are lost despite protein sparing, e. g. through carnitine shuttling, and individuals can die of starvation if the organism protein resources decrease by about 30 % (Bennett et al., 2007). Once they have learned to feed themselves, the young eat small fish, mollusks and aquatic arthropods (Beck et al., 2007; Gårdmark et al., 2012; Keszka et al., 2020; Lundström et al., 2010). Therefore, the post-weaning fast is a critical period for grey seal pups, when accumulated limited energy resources have to be allocated for different physiological processes and the acquisition of certain feeding and movement skills (Jenssen et al., 2010; Noren et al., 2005; Øritsland et al., 1985; Reilly, 1991). As mentioned earlier, depending on maternal input and growth conditions during early ontogeny, weaned pups may differ by as much as 20 kg in body weight during post-weaning fast. Therefore, smaller individuals with insufficient energy resources during post-weaning fast risk freezing or starving to death.

Activity of grey seal pups immediately after weaning are characterized by a very low levels, meaning that pups spend most of their post-weaning time resting (Haller et al., 1996; Kovacs, 1987b; Lydersen and Kovacs, 1999). Throughout lactation, they remain on land (unless exposed to water by tidal waves, storm surges, or ice melt) and spend approximately 70-80% of their

time sleeping or inactive (Haller et al., 1996; Kovacs, 1987b). During the post-weaning period pups remained less mobile, except the time spent in the water, the exploration behavior decreased over time (Kovacs, 1987b). As during lactation, play and exploratory behavior is limited to interactions with inanimate objects, sudden movements, and jumps that are not directed toward another (Haller et al., 1996; Kovacs, 1987b). It is interesting that even after weaning, grey seal pups rarely show any interest in social behavior. By limiting their mobility during suckling period, pups save energy resources and reduce metabolic rate, reduce the risk of separation from the mother and avoid aggression from other suckling mothers and breeding adult males (Haller et al., 1996; Kovacs, 1987a; Lydersen and Kovacs, 1999). However, there are limited amount of studies investigating when young begin to socialize, apparently due to a particularly aquatic lifestyle and long migrations in the first year of life (Bennett et al., 2010; Breed et al., 2006; Carter et al., 2017; McConnell et al., 1999; Robinson et al., 2017, 2015; Sjöberg and Ball, 2000; Wilson, 1974).

Grey seals are social polygynous mammals with sexually dimorphic social behaviors that can be observed not only during the breeding season, but also during social play outside the breeding season, as was mentioned in the first chapter. Although causes that lead to social behavior differences of grey seals during early development have not been investigated, studies, focusing mainly on of laboratory mammals, indicate sex steroid hormones as one of the main factors (Adkins-Regan, 2007; Amateau et al., 2004; Auger and Olesen, 2009; Meaney and Stewart, 1981; Olesen et al., 2005).

2.1.2. Steroids and their role in early physiological and social development

Steroids are four ring polycyclic lipid soluble molecules and derivatives of cholesterol (Goodman, 2022). Vertebrate steroid hormones belong to five classes: progestogens (progestins) such as progesterone with 21 carbon atoms, glucocorticoids such as cortisol and mineralocorticoids such as aldosterone with 21 carbon atoms, androgens such as testosterone with 19 carbon atoms, and estrogens such as estradiol with 18 carbon atoms (Ruiz-Cortes, 2012; Zubeldia-Brenner et al., 2016) (Fig. 2.1). Mineralocorticoids and glucocorticoids are sometimes referred as corticosteroids.

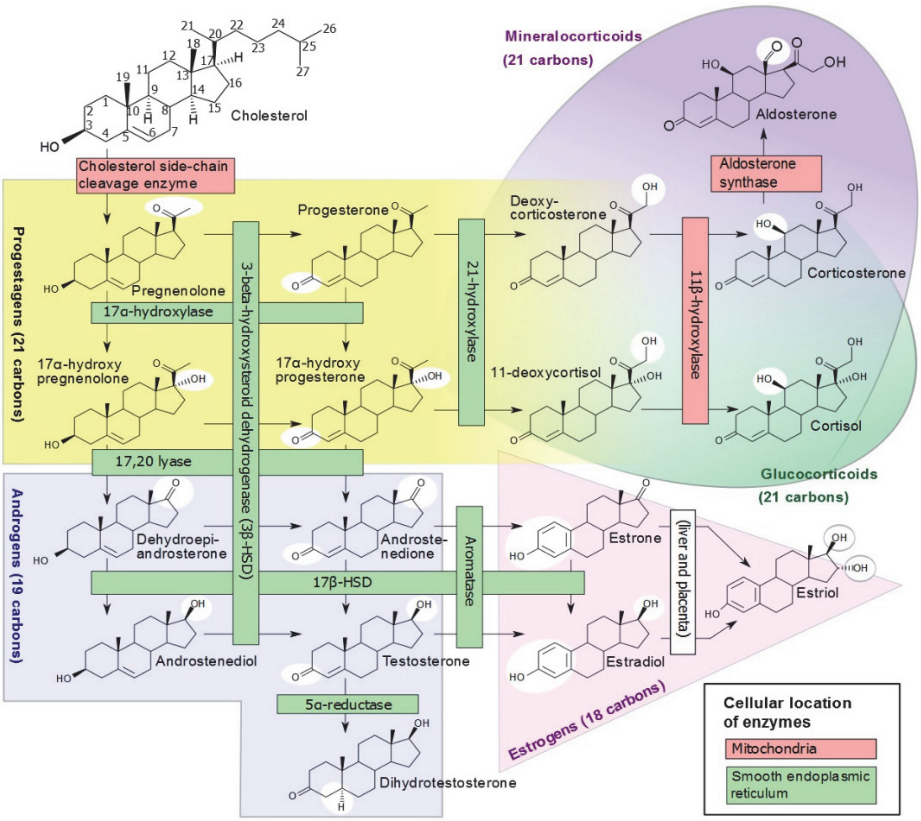


Fig. 2.1. Pathways of human steroidogenesis (from Häggström and Richfield, 2014)**.**

The first characteristic and common precursor of other steroids is pregnenolone which is derived from the membrane lipid cholesterol formed from squalene which itself is built from mevalonic acid (Kleine and Rossmanith, 2016). Cholesterol is transported in blood by low and high density lipoproteins (Ruiz-Cortes, 2012). Steroid synthesis depends on the speed of transport of free cholesterol from cytoplasm to mitochondria. The enzymatic step from cholesterol to pregnenolone (the common branch point for synthesis of progestins, corticoids, androgens, and therefore estrogens) by cytochrome P450 side-chain cleavage enzyme is the limiting step for steroidogenesis once cholesterol is inside the mitochondria (Zubeldia-Brenner et al., 2016). Progesterone (P4) is generated in the ovary, the adrenal gland, the placenta during pregnancy and the nervous system, in which it plays an important role as a neurosteroid. This steroid is the principal intermediate for

circulating androgens and estrogens (Zubeldia-Brenner et al., 2016) that will be discussed later. The largest part of circulating steroids is bound to blood proteins (globulins and albumins) and only around 10 % are free steroid which is the most active and potent part (Hamilton et al., 2022; Ruiz-Cortes, 2012).

The mechanisms of action for different steroid hormones are relatively similar in the different target tissues. However, the exact location of steroid receptors is still questionable. In some studies with low amounts of steroid hormones receptors of androgen, estrogen and progesterone are located in the nucleus, whereas receptors for glucocorticoid – in the cytoplasm (Mani et al., 2012), some other studies state that majority of steroid receptors are cytoplasmic (some even distributed on the cell membrane) till they bind the ligand and then they translocate to the nucleus to initiate genomic actions (Levin and Hammes, 2016). Steroid hormones move passively from the circulation and interstitial spaces across cell membranes, initiate their movement to nucleus and bind to response elements on target genes (Mani et al., 2012; Ruiz-Cortes, 2012; Zubeldia-Brenner et al., 2016).

2.1.2.1. Sex steroids

Androgens, estrogens and sometimes progestogens are classified as sex-steroids (Ruiz-Cortes, 2012). Estradiol (E) is the most biologically prevalent and active compound of a class of steroids called estrogens. Two other estrogens estrone (E1) and estriol (E3) are less potent. Estradiol is about 10 times as potent as estrone and about 80 times as potent as estriol in its estrogenic effect. Estrone is the predominant circulating estrogen during menopause and likely before maturity, while estriol is the predominant circulating estrogen during pregnancy. Estradiol is also present in males as an active metabolic product of testosterone and circulates in very low levels (14 - 55 pg/mL) that are roughly comparable to those of postmenopausal women (< 35 pg/mL) (Pedernera-Romano et al., 2006; Ruiz-Cortes, 2012; Zubeldia-Brenner et al., 2016). In females, estrogens and progestins are synthesized and secreted principally by maturing ovarian follicles, corpora lutea and during pregnancy, the placenta. Estradiol, and activity of the enzyme responsible for estradiol synthesis, P-450 aromatase, as well as estrogen receptors, are all at their highest levels in the brain either prenatally or during the first few days of life and then gradually decline to adult levels rodents and some primate species (McCarthy, 2008). Estradiol exerts potent and wide-ranging effects on the developing brain (Ruiz-Cortes, 2012).

Males develop in an environment of elevated testosterone (T) secreted by the fetal testes that acts to masculinize and defeminize brain structures, physiological processes and behaviors (Main et al., 2000; Ruiz-Cortes, 2012; Zubeldia-Brenner et al., 2016), like genital development (Main et al., 2000). T is produced by several tissues in the body – Leydig cells of the testes, thecal cells of the ovary, and cells in the reticularis region of the adrenals, and can be reduced to a more active metabolite – 5α-dihydrotestosterone (DHT) (Ruiz-Cortes, 2012). This process takes part mainly in the target tissues and in some, T and androstenedione (A4) can also be transformed into estrogens, such as 17β-estradiol (E) and estrone (E3). Androstenedione (A4), androstenediol (AN) and dehydroepiandrosterone (DHEA) are considered to be weak androgens and act as precursors to more potent steroids (Fig. 2.1).

Although sex steroid levels in the circulation are lower in pre-pubertal individuals than adults, they are still detectable and important mediators of behavior and energy partitioning, especially during early postnatal life (Bell, 2018). Testosterone (T), estradiol (E) and their active metabolites produce sex-specific effects on neural organization during prenatal and early postnatal periods and induce sex-specific activation of neural circuitry during adolescence of mammals and birds (see (Bell, 2018; Celec et al., 2015; Wallen and Baum, 2002; Wilson and Davies, 2007 for review and Konkle and McCarthy, 2011; Shulz et al., 2009 for original research). The effect of T and E on the brain leads to different social and sexual behaviors of males and females and impacts on their reproductive success (French et al., 2013; Klein et al., 1997; Place et al., 2002). For example, T plays a major role in activation of sexual behavior in adult males, while E maintains pregnancy in adult females (Norris, 2007a). Circulating blood T and E levels are also important for the formation of early life social behavior, such as social play in mammals species (Auger and Olesen, 2009; Meaney and Stewart, 1981).

As mentioned previously, sex steroids are high in concentrations immediately after birth in many mammal species (Amateau et al., 2004; Clarkson et al., 2016; Wong et al., 1992). These high concentrations are thought to work as an organization factor for future social behavior that is activated during puberty – during an activation period (Adkins-Regan, 2007; Auger and Olesen, 2009; McCarthy, 2008; Trova et al., 2021). Studies on rat social play behavior show that sexual differences in social play behavior arise due to different exposure of testosterone during the perinatal period and those male rats that were castrated early in the postnatal period show female typical frequency of play (Meaney

and Stewart, 1981). Conversely, administration of peripheral testosterone (Thor and Holloway, 1986), its metabolite dihydrotestosterone or insertion of testosterone implants on amygdala into neonatal female brain (Meaney and Stewart, 1981; Tönjes et al., 1987) can lead to masculinized social play levels in the future. The importance of testosterone for social play differentiation also shown through androgen receptors (ARs) that are known to play a major role in mediating the effects of testosterone on the organization of social play, as males exposed perinataly to AR antagonists, like flutamide or vinclozolin display reduced frequency of social play (Casto et al., 2003; Hotchkiss et al., 2003; Place et al., 2002). Immediately after birth and up to one day old male rat pups contain significantly higher estradiol concentrations in many brain regions except hypothalamus (Amateau et al., 2004). High doses (100 μg) of peripheral estradiol protagonist estradiol benzoate (EB), which according to the authors resembles male-typical levels of estradiol in the neonatal brain, appear to masculinize juvenile social play in females to male-typical levels (Olesen et al., 2005). The importance of estrogen receptors (ER) in differentiating social play has been showed in rat males that are insensitive to androgens due the testicular feminization mutation (Field et al., 2006). Despite having decreased social play levels compared to normal males, these males did play more frequently and with same male specific play components compared to females. Additionally, perinatal exposure to the environmental (bisphenol A) estrogen and synthetic (diethylstilbestrol) estrogens can masculinize play behavior in female and hypermasculinize play in males rats (Auger and Olesen, 2009; Dessì-Fulgheri et al., 2002) and non-human primates (Goy and Deputte, 1997; Wallen, 2005). Although both – androgen and estrogen – receptor systems are involved in organization of social play, it is considered that ER plays a more important role as it increases AR activity (McAbee and DonCarlos, 1999).

As discussed more broadly by Auger and Olesen (2009), possible convergence of neurotransmitters and steroid receptors on play development exists and dopamine appears to be one of the most important neurotransmitters for sexual differentiation in the brain and leads to sex specific behavioral differences in both male and female rats (Gonzales et al., 2000; Götz et al., 2009; Hull et al., 1984; Tönjes et al., 2009). Dopamine also appears to be important for sexual differentiation of social play behavior, as treatments of neonatal females with different dopamine receptor agonists will masculinize juvenile social play (Götz et al., 2009; Olesen et al., 2005; Tönjes et al., 2009). Because dopamine can alter gene expression within specific regions

of developing amygdala (an area important for social play) via ER, both these factors influence and control the organization of social play behavior (Olesen et al., 2005; Olesen and Auger, 2008). Measurement of circulating sex steroids in young animals may therefore be important in understanding behavioral differences within or between sexes (Bell, 2018; Jacklin et al., 1988; Kung et al., 2016), but is likely to be challenging because levels will be much lower than in adults (Norris, 2007b).

2.1.2.2. Corticosteroids

In mammals, endogenous cortisol and corticosterone have widespread functions within the body and their production and secretion are controlled by the hypothalamic-pituitary-adrenal (HPA) axis. Like sex steroids, glucocorticoids are produced from cholesterol in the mitochondria (Cain and Cidlowski, 2015) and in the cascade of their production, neural, cytokine and endocrine signals are received in the hypothalamus where corticotropin-releasing hormone (CRH) is released and stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) straight into the blood stream (Webster et al., 2002). In a response to circulating ACTH the *zona reticularis* and *zona fasciculata* of the adrenal cortex produces cortisol and corticosterone, and release them into the blood circulation (Cain and Cidlowski, 2017, 2015). Excess cortisol levels negatively feedback to the hypothalamus reducing the production of CRH and ACTH. Cortisol is the most active corticosteroid in most mammals (corticosterone in most rodents), which can be converted by two forms of 11β- hydroxysteroid dehydrogenases (11β-HSD) to inactive products like cortisone (corticosterone to 11-deoxycorticosterone in rodents) and back to an active form (Edwards and Burnham, 2001). Thus a cortisol – cortisone ratio is often measured for indication of appropriate function of 11β-HSD (Edwards et al., 1996). Resent research demonstrated that marine mammal blubber is a site of an active steroid metabolism and can interconvert cortisol and cortisone (Galligan et al., 2018b; Kershaw and Hall, 2016).

Corticosteroids are a fundamental part of appropriate stress responses, bone growth, maintain glucose homeostasis and temporarily suppress immune function in many mammals (Norris, 2007a; Raulo and Dantzer, 2018). However, prolonged high levels of cortisol can lead to serious immune system failures, have negative impact on reproductive performance through suppressed hypothalamic-pituitary-gonadal axis in adults, elevate blood glucose and induce insulin sensitivity, and can negatively alter social behaviour in young

individuals (Levine, 1993; Norris, 2007; Sapolsky, 2004; Spencer, 2017). For example, such stressors as malnutrition, social isolation, mother deprivation and other disturbances has an impact on social behaviour of young individuals and can lead to future serious social and reproductive disadvantages (Bowman et al., 2004; Hol et al., 1999; Levine, 1993; Van Den Berg et al., 1999; Ward, 1972). Both short and long term stress during perinatal and early postnatal developmental period which leads to increased cortisol concentrations have an effect to a so called "social phenotype" (Spencer, 2017). The neuroendocrine stress response and the resulting changes in circulating glucocorticoid "stress hormones" may indeed play a strong modulating role in animal personalities and behavioral syndromes both through their organizational (developmental stress) and activational (acute stress) effects on behavior (Raulo and Dantzer, 2018; Rossi et al., 2018). Studies with laboratory rats show that sustained increases in glucocorticoid levels may affect social behavior by either increasing the production of CRH that may reduce social behavior by activating fear-related brain circuitry (Schulkin et al., 2005) or by shifting the effects of CRF on neural circuitry involved in reward and promoting social aversion (Lemos et al., 2012). Additionally, similar experiments show that primary social relationships or lack of them significantly alter HPA axis responsiveness, where isolated or mother deprived individuals have reduced social behaviours, such as social motivation, lack of approach behaviour and social interaction, increased aggression, social avoidance and avoidance of new environment and stimuli (Levine, 2001; Lukkes et al., 2009; Von Frijtag et al., 2002; Weiss et al., 2004).

Recent in field experiments on grey seals showed that both males and females have constant individual differences in behavioral responses, as so called personality traits (Shuert et al., 2018; Twiss et al., 2012b, 2012a; Twiss and Franklin, 2010). A research of grey seal pups also showed that pups from more crowded areas that are related to higher rates of aggressive interactions are more aggressive towards conspecifics (Robinson et al., 2017), thus a possible factor for a constant behavioral difference to form. However, none of these studies looked at glucocorticoid levels as one of the factors affecting the development of the social behavior.

2.1.3. Endocrine disruptors in the wild

As mentioned previously, critical periods during early development are especially important for organizational action of steroid hormones that lead

to permanent establishment of sex specific behaviors, development of sexual genitalia and future potential of correct mating behavior for males and females. Gonadal, placental and maternal hormones that mammals are exposed during early development must be maintained within an appropriate range. Correct organization effect that leads to activation followed by high circulating steroid concentrations in puberty are permanent and act to hardwire the brain (Zubeldia-Brenner et al., 2016).

However, exposure to high levels of steroidal hormones during critical early developmental period disrupts normal endocrine function and decreases fertility in mammals including humans and can even be transmitted to following generations (Uzumcu et al., 2006; Zubeldia-Brenner et al., 2016). The molecules that mimic or block hormonal activity are known as endocrine disruptors (ED) (Zubeldia-Brenner et al., 2016). Regardless of the source of hormones or ED during this period, they would alter the normal development of the offspring due to a reprogramming of the genes.

Grey seals are prime sentinel species providing early warnings on potential negative natural and anthropogenic impacts on aquatic ecosystem and permitting better management of these impacts that affect both human and animal health (Baily et al., 2016; Bossart, 2011; Watkins et al., 2022). According to Fossi and Marsili (2003), fish-eating aquatic mammals may be extremely vulnerable to environmental disruptors (EDs) because of (a) their position in the food chain, (b) dependence on an aquatic/marine food web, (c) they live in areas influenced by industry and agriculture, and (d) their specific reproductive physiology. In addition, their unique fat reserves can serve as depots for anthropogenic toxins, such as polychlorinated organic pollutants (Fossi and Panti, 2018; Sonne et al., 2022). It is especially true in Baltic sea, where grey seals were almost extinct in 80's due to high organochloride pollution (Harding and Härkönen, 1999; Jensen et al., 1969; Olsson et al., 1994). Even though the population is increasing since the ban of dangerous persistent organochloride pollutants (POPs) such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) in agriculture, they are still detected in the waters with higher levels in the Baltic sea where the status of grey seals is still worse than that of the Atlantic populations (Jenssen, 2006; Sonne et al., 2020; Sørmo et al., 2009, 2005).

There are four types of organochlorine endocrine disruptors commonly found in aquatic mammals: environmental estrogens, environmental androgens, antiestrogens, and antiandrogens (Fossi and Marsili, 2003). These en-

docrine disruptors act by mimicking various natural steroids by binding to hormone receptors or influencing cell pathways (e. g. environmental estrogens and androgens), or by blocking and altering hormonal binding to hormone receptors (e. g. antiestrogens, antiandrogens) (Fossi and Marsili, 2003; Sonne et al., 2022) and some of them have a direct effect on corticosteroid receptors (Hamilton et al., 2022). High levels PCBs have impact on steroid concentrations in both sexes of marine mammals which can disturb the reproductive cycle (Ciesielski et al., 2017; Dietz et al., 2015; Haave et al., 2003; Oskam et al., 2003) and response to stress (Hamilton et al., 2022). There are differences in POP burden between different sexes – females usually have lower levels of POP, because of maternal transfer to pup through milk (Debier et al., 2003; Habran et al., 2013; Robinson et al., 2018). High fat milk contain high levels of lipophilic contaminants, resulting in exposures of POP in grey seal pups to similar concentrations as adult animals (Sørmo et al., 2003). Well-fed pups from the Baltic Sea had concentrations of POPs up to 10 times higher than pups from the Atlantic Ocean (Sørmo et al., 2003). Therefore it is important to identify steroid levels in grey seal pups to understand what steroids and at what particular developmental stages are most vulnerable by POPs.

2.1.4. Steroid assessment

It is important to use appropriate techniques and matrices for steroid measurement to assess population health in wildlife and individual health in rehabilitated and long term captive animals (Atkinson and Gilmarti, 1992; Mashburn and Atkinson, 2004; Oki and Atkinson, 2004; Petrauskas et al., 2006). Sample collection methods and hormone analysis procedures should be refined to reduce animal handling time and stress, and to simplify sample preparation procedures to obtain accurate and physiologically meaningful results. Blood samples are often used as the matrix of choice for analysis of steroid samples in pinnipeds. However, cortisol levels change rapidly in response to a range of stressors, making it challenging to obtain good baseline measures (Raulo and Dantzer, 2018). Samples taken up to 3 min. of length should cover baseline concentration of cortisol (Raulo and Dantzer, 2018). There are also sample matrix related aspects, such as lipemia, which can occur in suckling young, produces light scattering and hemolysis produces strong absorbance at 450 nm, both of which interfere with colorimetric enzyme linked immunosorbent assays (ELISA). In addition, the presence of other nonspecific 'matrix' effects' can interfere with antibody binding and prevent accurate

quantification (Makin et al., 2010; Wong et al., 1992). The use of protein-rich plasma is not recommended in ELISA without extraction, since proteins can negatively affect binding protein availability (Holder et al., 2010). Removal of lipids and other interacting substances is therefore necessary in many cases, and extraction of steroids from the sample is often recommended for young animals (Makin et al., 2010). Due to differences in adrenal cortex activity, neonates can also produce additional steroid metabolites that cross-react with some immunoassay antibodies and can make quantification challenging (Makin et al., 2010).

New technologies have enabled the detection of steroids in many matrices, from blood to body hair (Koren et al., 2002; Norris, 2007a). Some alternatives to plasma sampling for steroid analysis have been tested in captive and wild pinnipeds, including fecal (Mashburn and Atkinson, 2004; Petrauskas et al., 2006), hair (Keogh et al., 2020; Meise et al., 2016) and blubber (Kershaw and Hall, 2016) sampling. These matrices present different advantages and challenges in terms of collection, sample processing, interpretation and utility to answer different questions. Fecal samples from Steller sea lions (*Eumetopias jubatus*) were collected in captivity (Petrauskas et al., 2006; Petrauskas and Atkinson, 2006) and in the wild (Mashburn and Atkinson, 2004) and glucocorticoid levels in this matrix showed good correlation with seasonality and behavior. However, fecal sample collection can be challenging in the wild due to the aquatic lifestyle of these species, where part of samples can be lost, diluted or contaminated. Also, it is often difficult to identify which individual produced the sample. Hair and blubber attenuate cortisol spikes observed in blood and provide good matrices for differentiating physiological and environmental states (Keogh et al., 2020; Kershaw and Hall, 2016; Meise et al., 2016), but hair can reflect very extreme and long lasting changes of steroid concentrations (Keogh et al., 2020; Meise et al., 2016), while blubber sampling requires a biopsy to be taken and measurements from this matrix might not map to short term behavioral changes (Kershaw and Hall, 2016). Moreover, all three methods mentioned here require substantial preprocessing time before analysis.

There is a growing interest in saliva sampling for steroid analysis because it can avoid the need for venipuncture (Chiappin et al., 2007; Gröschl, 2009; Hellhammer et al., 2009) and is a simpler matrix: only free steroids, representing the biologically active fraction of steroid hormones, reach the saliva from the bloodstream (Gröschl, 2008). In addition, unconjugated steroids, such as

salivary cortisol diffuse freely through the salivary gland acinar cells, showing little change at low and high extremes of salivary flow rate (Vining et al., 1983) and good correlations with free steroids in blood (Wood, 2009), even though there is typically a lag of 2 to 10 min. between salivary and blood cortisol levels (Hernandez et al., 2014; Kirschbaum and Hellhammer, 1989). Salivary steroid concentrations may also vary less than plasma levels, making them more suitable to obtain longer term average levels (Hernandez et al., 2014).

2.1.5. Blood and saliva use in pinniped steroid research

The full list of steroid studies performed in pinniped species is presented in Table S1. Most pinniped steroid research is performed with adult animals using radioimmunoassay (RIA) as an analysis method, followed by liquid chromatography mass spectrometry (LC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS), chemiluminescence immunoassay (CLIA) and electro chemiluminescence immunoassay (ECL) where both – blood plasma or serum – were used as a matrix. Enzyme immunoassay use was reported in just a few studies (Table S1).

Although cortisol has been measured in young grey seals in the context of stress and energy balance (Bennett et al., 2013, 2012; Nordøy et al., 1990), there have been no studies on androgens and estrogens in juveniles of this species. Studies in other young pinnipeds have shown that steroid concentrations change during early development, e. g. studies in Northern elephant seal pups showed that both E and T levels as well as C increase with fasting (Ortiz et al., 2003; Sherman-Cooney et al., 2005). Measuring steroid concentrations in young animals will help to elucidate the impact they have on the early physiological and behavioral development of grey seals, and whether they contribute to the observed sex-specific differences in the first-year survival (Hall et al., 2001) and behavior in this species (Breed et al., 2009; Carter et al., 2019, 2017; Survilienė et al., 2016; Trippel et al., 1996; Twiss et al., 2012b). However, their plasma presents challenges identified above: although grey seal pup plasma contains only around 1,71-2,63 mg/ml triglycerides (Iverson et al., 1995; Iverson & Bowen, 1995), which is below levels recommended for typical steroid ELISAs (e.g. 45 mg/ml for testosterone, 30 mg/ml for estradiol and 90 mg/ml cortisol (Ibl International, Hamburg)), suckling pups often have lipemic blood (KB pers. obs.). Grey seal pup plasma also contains ~70 g/l of protein during the first 6 weeks of life (Erokhina and Kavtsevich, 2018), which may reduce ELISA performance, therefore preprocessing would be encouraged.

Salivary samples from captive pinnipeds have been used to measure testosterone (Harmon, 2001; Theodorou and Atkinson, 1998), estrone sulfate and progesterone (Pietraszek and Atkinson, 1994) and cortisol (Nagel et al., 2022) require less processing and correlate well with matched blood steroid measurements, such that they may be useful in understanding behavioral correlates of endocrine status. However, wild animals may have oral injuries or may struggle during collection leading to anesthesia or restraint (Nagel et al., 2022), and blood-contaminated saliva leads to elevated steroid values (Granger et al., 2004; Toone et al., 2013). Moreover, commercial ELISA kits usually require up to 100 µl of saliva for one steroid analysis – more than typically needed in a plasma ELISA, because steroid levels are up to 10 times lower in saliva (Gröschl, 2009).

2.1.6. Rationale

Steroids are important natural chemicals responsible for various physiological and behavioral changes in vertebrate species, and especially during early development and their levels can be affected by endocrine disruptors. This effect could lead to physiological and behavioral changes. However, nothing is known about the levels of sex steroids in young grey seals during their suckling and post-weaning fast periods. ELISA is one of the easiest, least invasive and efficient method to analyze steroid hormones in different matrices, however it has not been used for grey seal steroid research and has to be validated for every commercial kit. Comparisons between different methods are useful for confirming the biological validity of the method. In addition, it is important to evaluate the feasibility, quality and effectiveness of saliva sample collection against the more usual blood sampling from wild pinnipeds, especially in young animals for which sex steroid levels in saliva may be particularly low. Here an attempt to provide some insight into the relationship between steroid concentrations and behavior was made.

Main aim:

To investigate changes of steroid hormones in different matrices of grey seal pups during suckling and post-weaning fast periods by using different analysis methods, and link steroid concentrations to pup behavior.

Main objectives:

1. To assess the suitability of saliva for analysis of three steroid hormones (estradiol, testosterone and cortisol) by investigating the success of

sample collection including approximate volume obtained and compare the sample preparation and analysis efficiency in the laboratory.

2. To assess performance of commercially available ELISA kits for saliva and plasma measurements, steroid (estradiol, testosterone and cortisol) levels and compare the resolution of two matrices.
3. To assess the comparability of plasma steroid concentrations measured with ELISA and two alternative methods (RIA, UPC_2-MS/MS).
4. To examine the interactions between grey seal pup behavior and steroid concentrations during suckling and post-weaning fast periods.

2.2. MATERIALS AND METHODS

2.2.1. Animal location and sampling

Samples were collected from grey seal pups that formed part of a long term study of grey seal reproduction during the breeding seasons (October-December) of 2012, 2016 and 2017 on the Isle of May, Scotland (56◦11'N, 02◦33'W). Blood samples were collected during daylight between 8:02 to 17:10 GMT (mean ± SD = 13:57 ± 2:20). 127 samples from 44 pups (24 males (n = 74) and 20 females (n = 60)) in 2012, 2016 and 2017 on up to four occasions (four nutritional stages: S1 – early suckling, S2 – late suckling, W1 – early weaning, W2 – late weaning) during suckling and the post-weaning fast were analyzed. Samples were taken when animals were 4 to 38 days old (details of animal age at sampling are provided in Table S2). Animals of stage 2-3, assessed based on a body shape, umbilicus and pelage (Kovacs and Lavigne, 1986a), were targeted for the initial sampling (Supplementary material Fig. S1). Where birth date was not known, the age was estimated from body mass and appearance of the pup at first capture (Pomeroy et al., 1999) and thus age varied within different nutritional stages (Table 2.1).

Table 2.1. Grey seal female (F) and male (M) body mass (mean ± SE, kg), measured in different years (2012, 2016, 2017) during different nutritional stages (S1, S2, W1, W2). Pup age (min-max, mean ± SD, days) is given for every nutritional stage.

	S1 2-11 days of age (5,78 ± 1,97)	**S2 13-20 days of age (15,8 ± 1,52)**	**W1 21-30 days of age (25,5 ± 2,63)**	**W2 32-40 days of age (35,5 ± 2,6)**
Pup Body mass (kg) 2012	24,86 ± 0,67 F: 24,94 ± 3,2 M: 24,8 ± 3,28	42,68 ± 1,1 F: 42,35 ± 4,3 M: 43 ± 4,69	41,25 ± 1,26 F: 41,5 ± 4,77 M: 40,97 ± 5,4	37,38 ± 1,27 F: 36,74 ± 5,1 M: 38,28 ± 3,53
Pup Body mass (kg) 2016	20,95 ± 2,59 F: 20,73 ± 2,6 M: 21,2 ± 2,87	40,54 ± 4,12 F: 39,53 ± 4,3 M: 41,3 ± 6	40,77 ± 7,41 F: 35,73 ± 4,12 M: 45,8 ± 4,65	36,57 ± 4,78 F: 39,8 M: 35,92 ± 5,04
Pup Body mass (kg) 2017	23,25 ± 4,12 F: 22,1 ± 3,3 M: 24,56 ± 5	39,97 ± 8,51 F: 38,1 ± 5,52 M: 40,72 ± 9,9	41,25 ± 1,26 F: 42,07 ± 8,42 M: 40,53 ± 8,8	36,25 ± 7,49 F: 39,13 ± 8,9 M: 35,3 ± 7,74
Mean pup Body mass (kg)	23,62 ± 3,54 F: 23,31 ± 3,4 M: 23,9 ± 3,7	41,53 ± 5,62 F: 41,05 ±4,41 M: 41,91±6,5	41,06 ± 6,37 F: 40,22 ± 6,18 M: 41,74 ± 6,7	37,02 ± 5,58 F: 37,93 ± 5,92 M: 36,71 ± 5,41

Of those collected 122 samples were included in further analysis presented in the Results section, while others were used for optimization and trials of steroid analysis. Details on samples collected for each steroid and matrix from each pup during different nutritional stages and samples analyzed are provided in the Supplementary Material Table S2.

2.2.2. Sample collection and processing

Blood collection and processing. Animals were manually captured, blood sampling area cleaned and then anesthetized before they were weighed and before any sampling was performed. Mothers were anesthetized using intramuscular injection from a blow dart (Zoletil $_{100}$, Virbac, France) and capture took place once the anaesthesia had taken effect approximately 10 min. later. Until then the capture team remained 20-30 meters away from a mother-pup pair. Mothers and pups were then captured simultaneously. All pups (totally 107 samples), except 15 samples from 10 individuals taken at different ages (Supplementary Material Table S2), received approximately 0,1 mg/kg, i.v. of anesthetic (Zoletil $_{100}$, Virbac, France), a safe dose for animals of this age class and effective for the sampling regime, to obtain blubber samples for another study measuring gene expression or measuring the effect of POP on blubber layer (Bennett et al., 2021, 2015; Robinson et al., 2018). The four animals that were not anesthetized did not have blubber or saliva samples taken and were sampled only for blood. Exact duration of blood collection was estimated only for 7 samples collected in 2016 and took up to 2 min. (1,71 ± 0,49 min.). This also agreed with timing from blood collection procedure that was performed as in Bennett et al. (2012), in which blood was obtained within two minutes from initial physical contact with the animal and is considered to represent baseline cortisol concentrations (Nagel et al., 2022; Raulo and Dantzer, 2018). Blood was collected from the extradural vein into a sterile 10 ml potassium ethylenediaminetetraacetic acid (EDTA) Vacutainer (Becton Dickinson, Oxon, UK). Blood samples were centrifuged in a swing-out bench top centrifuge at 2000 g for 15 min. The plasma was then drawn off using glass Pasteur pipettes, divided in 0,5 or 1,5 ml aliquots, immediately frozen and stored at -20°C (for 2012) or -80°C (2016, 2017) until extraction and analysis.

Saliva collection and processing. Saliva samples were collected from grey seal pups in 2012 and 2017 on the Isle of May, UK. Saliva sampling was attempted during every blood sampling when animals were anaesthetized,

since collecting saliva samples from mobile and aggressive unanesthetized pups was considered too stressful to the animal and risked injury for the handler. Saliva samples were taken with inert polymer, cylindrical swabs (8 mm × 125 mm) designed for saliva sample collection in humans (Salimetrics, UK). The swab was inserted into the side of the mouth while the animal was anesthetized, which allowed sample collection without additional restraint (not more than 10 minutes). Thus the duration of saliva sample collection was up to 5 times longer (< 10 min.) than that for taking blood (< 2 min.). One end of the swab was held and the animal was allowed to move and chew it. Before removal, the swab was used to collect saliva from the corners of the mouth. The mouth area and gums were checked prior to, during and after the saliva sampling procedure to avoid blood contamination. No samples were collected when signs of blood or abrasions were visible inside the mouth or around the gums. Any other particles of grass or dirt, if there were any, were not rinsed away to avoid diluting the saliva sample. The sample was discarded if any traces of blood on the polymer swab were observed during collection procedure and was not observed during the initial checking. Reasons for not collecting the sample were either blood contamination or low saliva volume (so called “dry mouth”). These two possibilities were not differentiated and were recorded as “not collected”. The swab was then transferred to a 2 mm sterile syringe, which was used to squeeze the saliva out of the swab into a 2 ml collection tube (Salicap, IBL International, Germany). The amount of saliva recovered was estimated from 0,1 ml gradations on the collection tubes. Blood and saliva samples were stored at ambient temperatures after collection for an average of 2 hours 9 min. (from 25 min. to 8 hours 49 min.) before they were processed.

2.2.3. Ethical statement

All sample collection was performed by personal license holders/ designated competent personnel under UK Home Office license numbers PPL 60/4009 (2012-2013) and PPL 70/7806 (2016-2017). This work received ethical approval from the University of St Andrews Animal Welfare and Ethics Committee (AWEC) and was performed in compliance with Animal (Scientific Procedures) Act (ASPA) 1986 and the EU directive on the protection of animals used for scientific purposes (2010/63/EU).

2.2.4. ELISA analysis

2.2.4.1. Plasma sample preparation and steroid extraction

For ELISA plasma EDTA samples were extracted according to the Steroid Liquid Sample Extraction Protocol provided by Arbor Assays® (Michigan, USA), which is recommended by ELISA kit various manufacturers (Kumar, 2020) and validated for prepubertal human serum using EIA and RIA (Ankarberg-Lindgren et al., 2001; Norjavaara et al., 1996; Raivio et al., 2001). Briefly, 300 µl of the sample were extracted with 1,5 ml of diethyl ether (to 1:5 ratio) by vortexing the mixture for 10 min. followed by a 10 min. centrifugation at 3000 g at room temperature and freezing the liquid in –80^{0}C for an easier separation of the top layer of organic solution. The top of the liquid layer was separated into a separate polypropylene 2 ml vial. The procedure was repeated three times to maximize the extraction efficiency. Collected material was then desiccated to dryness in vacuum Eppendorf concentrator 5301 (Hamburg, Germany) and frozen at -20˚C (2012) or -80˚C (2016, 2017) till analysis. Samples were thawed on the day of the ELISA, reconstituted in 300 µl of steroid free serum (Ibl-International, Hamburg, Germany) and vortexed for 30 min. at room temperature to allow any dry components to dissolve and mix completely, centrifuged for 10 min. at 3000 g and the supernatant used for ELISA.

2.2.4.2. Saliva sample preparation

Saliva samples were heated at 60˚C for 1 h prior to ELISA to denature any interfering substances (such as mucus and proteins that can affect light absorption etc.) according to the manufacturer's (Ibl-International, Hamburg, Germany) recommendations. Samples were left to cool to room temperature and centrifuged at 3000 g for 10 min. prior to ELISA.

2.2.4.3. Commercial ELISA kit validation and sample analysis

Commercially available kits by IBL-International (Tecan from 2019, Hamburg, Germany) were used for analysis of TB [Cat. No. RE52151], TS [Cat. No. RE52631], EB [Cat. No. RE52041], ES [Cat. No. RE52601], CB [Cat. No. RE52061], CS [Cat. No. RE52611]. The automatic ELx50 microplate washer (Biotek Instruments, Netherlands) was used for plate wash. ELISA kits were used according to manufacturer's protocols without any modifications. Light absorption was measured with microplate optical density reader ELx800™ (BioTek, Netherlands) and analyzed using program Gen5 ELISA ver. 1.11.5 (BioTek Instruments, Netherlands). The standard curve was

drawn using nonlinear 4 parameter regression. Sensitivity range reported by the manufacturer for steroids was 0,12 - 16 ng/ml for plasma testosterone (TB), 2 - 760 pg/ml saliva testosterone (TS) analyzed in 2014 and 2-900 pg/ml analyzed in 2017, 9,7 – 1000 pg/ml for plasma 17β-estradiol (EB), 0,4 - 100 pg/ml for saliva 17β-estradiol (ES), 2,46 – 800 ng/ml for plasma cortisol (CB), 0,015 – 4 μg/dl for saliva cortisol (CS). Lowest detectable level reported by manufacturer in ES – 0,4 pg/ml, TS – 2 pg/ml, CS – 0,005 μg/dl, and in EB – 10,6 pg/ml, TB – 0,07 ng/ml, CB – 2,46 ng/ml.

Each kit was validated for use with saliva and plasma from grey seal pups. All standards and samples were analyzed in duplicate within any given plate. *Precision* of commercial ELISA kits was determined by investigating intra-assay (intra-assay CV) and inter-assay (inter-assay CV) coefficients of variation. Assay validation and sample measurements for plasma samples were performed across two plates, which were analyzed over two consecutive years 2014 (Plate 1) and 2015 (Plate 2). Inter-assay CV of plasma between plates was estimated using two samples each measured on the two plates. All saliva samples were measured within one plate in both years – 2014 and 2017, thus only intra-CV is reported. To estimate the *accuracy* of each kit, pooled plasma and pooled saliva samples were each diluted with the zero standard provided by the manufacturer to 0,75, 0,5 and 0,25 of raw concentration. This dilution range was chosen to ensure the measurements remained within the limits of the standard curve. The concentrations in the dilutions were then compared with expected concentrations from the pooled samples. The pooled samples were spiked 1:1 with low and high standard provided by the manufacturer to obtain recovery values. Recovery rates were measured by comparing expected vs. observed concentrations.

In total 121 samples were analyzed (2012: n = 60, 2016: n = 30, 2017: n = 31) (Table 2.2). Since the concentrations of steroids, especially EB and TB varied a lot between the plates, in plates 2014 and 2015 there were no samples below the limit of detection (<LOD), but there were more of such samples from 2016 (Plate 3) [majority of TB (n = 24)] and from 2017 (Plate 4) (EB (n = 1) and TB (n = 13)) and they were discarded from any of the analysis. There were also concentrations that were below the limit of quantification provided by the manufacturers protocols (LOQ) in Plate 2 (TB = 12), Plate 3 (EB = 5, TB = 3 from 6 remaining samples) and Plate 4 (EB = 22, TB = 15). Therefore in this study concentrations that, according the light absorption analysis program, were above LOD, even though below the values indicated by the manufacturer, were included. Samples that had concentrations below LOQ and below LOD are mentioned in the Table S2 (Supplementary material).

Table 2.2. Number of grey seal pup and female saliva (ES, TS, CS) and plasma (EB, TB, CB) samples analyzed within different plates (years) and nutritional stages.

	Plate number (year)	**S1**	**S2**	**W1**	**W2**
Number of different **saliva** samples analyzed for different steroids (N = 53 - 61)	Plate 1 (2014) Samples from 2012 N = 31-37	ES – 7 (F=3, M=4) TS – 8 (F=4, M=4) CS – 10 (F=4, M=6)	ES – 6 (F=2, M=4) TS – 5 (F=1, M=4) CS – 6 (F=2, M=4)	ES – 8 (F=4, M=4) TS – 8 (F=4, M=4) CS – 10 (F=6, M=4)	ES – 10 (F=6, M=4) TS – 11 (F=6, M=5) CS – 11 (F=6, M=5)
	Plate 2 (2018) Samples from 2017 N = 22-24	ES – 3 (F=2, M=1) TS – 4 (F=2, M=2) CS – 3 (F=2, M=1)	ES – 4 (F=1, M=3) TS – 4 (F=1, M=3) CS – 4 (F=1, M=3)	ES – 8 (F=3, M=5) TS – 9 (F=3, M=6) CS – 9 (F=3, M=6)	ES – 7 (F=3, M=4) TS – 7 (F=3, M=4) CS – 7 (F=3, M=4)
Number of different **plasma** samples analyzed for different steroids (N = 121)	Plate 1 (2014) Samples from year 2012 N = 35	EB – 11 (F=7, M=4) TB – 11 (F=7, M=4) CB – 11 (F=7, M=4)	EB – 10 (F=5, M=5) TB – 10 (F=5, M=5) CB – 10 (F=5, M=5)	EB – 7 (F=2, M=4) TB – 7 (F=2, M=4) CB – 7 (F=2, M=4)	EB – 5 (F=3, M=2) TB – 5 (F=3, M=2) CB – 5 (F=3, M=2)
	Plate 2 (2015) Samples from a year 2012 N = 25	EB – 8 (F=2, M=6) TB – 8 (F=2, M=6) CB – 8 (F=2, M=6)	EB – 6 (F=4, M=2) TB – 6 (F=4, M=2) CB – 6 (F=4, M=2)	EB – 8 (F=5, M=3) TB – 8 (F=5, M=3) CB – 8 (F=5, M=3)	EB – 4 (F=2, M=2) TB – 4 (F=2, M=2) CB – 4 (F=2, M=2)
	Plate 3 (2018) Samples from a year 2016 N = 30	EB – 11 (F=6, M=5) TB – 11 (F=6, M=5)	EB – 7 (F=3, M=4) TB – 7 (F=3, M=4)	EB – 6 (F=3, M=3) TB – 6 (F=3, M=3)	EB – 6 (F=1, M=5) TB – 6 (F=1, M=5)
	Plate 4 (2018) Samples from a year 2017 N = 31	EB – 9 (F=4, M=5) TB – 9 (F=4, M=5) CB – 9 (F=4, M=5)	EB – 6 (F=2, M=4) TB – 6 (F=2, M=4) CB – 6 (F=2, M=4)	EB – 9 (F=3, M=6) TB – 9 (F=3, M=6) CB – 9 (F=3, M=6)	EB – 8 (F=4, M=4) TB – 8 (F=4, M=4) CB – 8 (F=4, M=4)

Cross reactivity with other substances reported by the manufacturer is: for ES and EB – Estrone (6,86 %), Estriol (2,27 %); for CS and CB – prednisolone 30 %, 11-Desoxy-Cortisol – 7 %, Cortisone – 4 %; for TS - 11β-Testosterone (4,22 %), 11α-Testosterone (3,59 %); for TB - 11β-Testosterone (8,67%), 11α-Testosterone (3,24 %).

Saliva was collected in different volumes (Supplementary material Fig. S2). When a partial saliva volume was collected and was below 100 µl, the cortisol assay was prioritized since it proved to be more reliable during preprocessing, required less sample (2 × 25 µl) than the estradiol kit (2 × 50 µl) and allowed results to be included in additional stress related studies. When partial saliva volume collected allowed analysis of two steroids, testosterone was chosen as the second option, because, like cortisol, it required a lower volume of saliva (2 × 25 µl) for the assay than estradiol kit (2 × 50 µl). As a result, the number of samples analyzed differs between steroids and is lowest for estradiol.

2.2.5. Plasma estradiol and testosterone analysis with RIA

The radioimmunoassay analysis (RIA) of 17β-estradiol (EB_RIA) and testosterone (TB_RIA) was performed in the Department of Biology, NTNU (Norwegian University of Science and Technology, Trondheim, Norway). Plasma EDTA samples were analyzed for total unconjugated 17β-estradiol and testosterone using commercial radioimmunoassay kits (MP Biomedicals, California, USA) according to manufacturer protocols (ImmuChem Testosterone DA, 07814891.2, Q15-042, A15-005 (3/16) and ImmuChem 17βEstradiol CT, 07815381.6, Q16-031, A16-003 (2/17)). Briefly, for the analysis of EB_RIA, 100 µL of serum was added to antibody coated tubes followed by 1 mL of radioactive 17β-estradiol ^{125}I tracer. Samples were incubated for 1 hour at 37 °C. The solution was decanted and tubes were allowed to air dry before 17β-estradiol final determination using γ-scintillation counter (Cobra Auto-Gamma, model 5003, Packard Instrument Co., Dowers Grove, IL, USA). For the TB_RIA assay, 50 µL of sample was added to the test tubes, followed by adding 100 µL of sex hormone binding globulin inhibitor (SBGI) solution (used to block testosterone binding to SBG during the assay) and 500 µL of radioactive testosterone ^{125}I tracer. Thereafter, 500 µL of anti-serum was added and samples were vortex-mixed, followed by incubation at 37 °C for 120 minutes. After incubation, 100 µL of second antibody was added, the samples were mixed and incubated at 37°C for additional 60 minutes. After incubation, all sampled were centrifuged for 15 min (1000 g),

supernatant decanted and antibody–antigen testosterone complexes determine by γ-scintillation counting.

Calibration curves and concentrations of EB_RIA and TB_RIA in the samples were calculated by the embedded software (SpectraWorks™ Spectrum Analysis Software, Packard Instrument Company, Connecticut, USA) in the γ-scintillation counter. The quality control/quality assurance (QC/QA) of measurements was monitored by the analysis of the standard reference material, Lyphocheck (R) Immunoassay Plus Control Levels 1, 2 and 3 (BioRad; California, USA). Samples were analyzed in triplicates (n = 3) and intra-assay coefficient of variation was < 8,5 % and 12,6 % for EB_RIA and TB_RIA respectively. Limits of detections (LODs) were set in accordance with those reported in the kit protocols a 22,8 pg/mL for EB_RIA and 0,1 ng/mL for TB_RIA. Cross reactivity with other substances reported by the manufacturer is with Estrone (6,2 %), Estriol (1,45 %) and other substances < 0,01 % for EB_RIA, and with 5α-Dihydrotestosterone (3,4 %), 5α-Androstane-3β, 17β-diol (2,2 %), 11-Oxotestosterone (2 %) and others < 1 % for TB_RIA.

In total 31 grey seal pup samples (Table 2.3) from the 2017 sampling season were analyzed using RIA method. All EB_RIA concentrations were above the limit of detection (LOD). However, only 6 samples TB_RIA were above LOD.

Table 2.3. Number of grey seal pup (female – F and male – M) samples analyzed within different nutritional stages (S1 – early suckling, S2 – late suckling, W1 – early post-weaning fast, W2 – late post-weaning fast period).

Nutritional stage	**S1**	**S2**	**W1**	**W2**
Collected in 2017, analyzed in 2021 N = 31	EB_RIA = 8 (F = 4, M = 4) TB_RIA = 3 (F < LOD, M = 3)	EB_RIA = 7 (F = 2, M = 5) TB_RIA = 1 (F = 1, M < LOD)	EB_RIA = 9 (F = 3, M = 6) TB_RIA = 2 (F = 2, M < LOD)	EB_RIA = 7 (F = 3, M = 4) TB_RIA M, F < LOD

2.2.6. UPC_2-MS/MS analysis

In total 31 grey seal pup samples from the 2017 sampling season were analyzed (Table 2.4). Nineteen steroids were determined at the Department of Chemistry, NTNU (Norwegian University of Science and Technology, Trondheim, Norway) using ultraperformance convergence chromatography tandem

mass spectrometry method (UPC_2-MS/MS). 19 analytical standards were used for the analysis – dihydroepiandrosterone (DHEA), androstenedione (AN), androstenediol (A5), testosterone (TS), 5α-dihydrotestosterone (DHT), 11-ketotestosterone (KetoTS), 11-deoxycorticosterone (DOC), 11-deoxycortisol (11-deoxyCOR), aldosterone (ALDO), corticosterone (COS), cortisol (COR), Cortisone (CORNE), pregnenolone (PREG), 17α-hydroxypregnenolone (17α-PREG), progesterone (P4), 17a-hydroxyprogesterone (17α-OHP), estrone (E1), 17β-estradiol (E2) and 17α-estradiol (17α-E2) – were purchased from Cerilliant (Texas, USA). Isotopically labelled internal standards, cortisone-$^{13}C_3$ (2,3,4-$^{13}C_3$-CORNE), dihydrotestosterone-$^{13}C_3$ (2,3,4-^{13}C-DHT), 17a-hydroxyprogesterone $^{13}C_2$ (2,3,4-$^{13}C_2$-17 α-OHP) and 17β-estradiol $^{13}C_2$ (^{13}C-E2)) were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Individual stock solutions were prepared in methanol (MeOH) (Merck, Damstadt, Germany) and stored at -20°C, with the exception of DHT, which was stored at -80°C. The calibration and working standards were prepared with MeOH through serial dilution. All standard solutions were stored at -20°C.

HybridSPE$^{(R)}$Phospholipid 30 mg/L (Sigma–Aldrich; Steinheim, Germany) cartridges were used for the isolation of the target steroid hormones. MeOH with 0.1 % ammonium formate (w/v) as precipitation agent was used for steroid extraction according to the following method with minor modifications. Aliquots of 150 µL of the plasma sample were transferred into a 15 mL PP tube (VWR International AS, Oslo, Norway) and 450 µL of precipitation agent was added. Samples were vortex-mixed and centrifuged (at 1968 g for 10 min.) at room temperature. The HybridSPE® cartridges were initially washed with either 1 mL MeOH before the supernatant was passed directly through at a flow rate of approximately 1 mL/min to amber glass liquid chromatography (LC) vials (1.5 mL) (VWR International AS, Oslo, Norway) and analyzed directly using UPC_2-MS/MS. Chromatographic separation was carried out on a Waters ACQUITY UltraPerformance Convergence chromatographic (UPC_2) system (Milford, MA, USA) coupled to a triple quadrupole (QqQ; Xevo TQ-XS) mass spectrometer (UPC_2-MS/MS) with a Zspray ESI ion source (Waters, Milford, U.S). Separation of steroid hormones was achieved on a ACQUITY CSH Fluoro-Phenyl column (3,0 x 100 mm, 1,7 µm) in ESI positive mode. For the analysis of steroid hormones, the gradient program was according to de Kock et al. (2018): modifier 2 % (0,5 min), 2-17 % (2,5 min), hold 0,5 min, back to 2 % (0,5 min.) and hold 1 min for a total run time of 5 min., where

the modifier was methanol: isopropanol (MeOH: IPA, 1:1) with 0,1 % formic acid. The electrospray ionization voltage was +2.8 kV in positive mode. Nitrogen was used as both the desolvation and cone gas, at a flow rate of 1000 and 150 L/h, respectively. The desolvation and source temperatures were 500 and 150°C, respectively. The specific MS/MS parameters are presented Table S3. All chromatographic data were acquired and processed using Intellistart, MassLynx and TargetLynx software packages (Waters, Milford, U.S.), with further processing in Excel (Microsoft, 2018).

It was possible to determine concentrations of 15 out of 19 analyzed steroids (Table 2.4). No signal was detected for E2, 17α-E2 and 17α-PREG. The instrumental LOQ was set to the lowest concentration of the concentration detected in the linear range of the calibration curve, and the LOD was estimated as LOQ/3. Thus even though a signal was detected for ALDO, after calculations from the calibration curve and taking into account blank values all concentrations appeared < LOQ. Moreover, this method allowed the determination of values of all samples only for 3 steroids – COR, CORNE and COS.

Table 2.4. Number of samples (N) analyzed as well as number of samples <LOQ for different steroids for every nutritional stage.

	S1	**S2**	**W1**	**W2**	**LOQ**	**LOQ/2**
N of samples analyzed:	**9**	**7**	**9**	**6**	**ng/ml**	**ng/ml**
N of samples < LOQ:						
Cortisol (COR)	-	-	-	-	0,33	N/A
Cortisone (CORNE)	-	-	-	-	0,33	N/A
corticosterone (COS)	-	-	-	-	0,33	N/A
11-deoxycortisol (11-deoxyCOR)	4	-	1	3	0,66	N/A
11-deoxycorticosterone (DOC)	3	4	6	5	0,33	N/A
Pregnenolone (PREG)	4	2	6	1	3,33	1,67
Progesterone (P4)	1	1	5	2	0,33	0,17
Androstenedione (AN)	6	6	7	4	0,33	0,17
Androstenediol (A5)	4	2	6	2	3,33	1,67
Testosterone (TS)	9	1	4	-	0,33	0,17
11-ketotestosterone (KetoTS)	8	5	9	4	0,33	0,17
5α-dihydrotestosterone (DHT)	1	1	5	2	0,33	0,17
Dihydroepiandrosterone (DHEA)	7	4	7	5	3,33	1,67
17α-hydroxyprogesterone (17α-OHP)	4	3	7	4	0,33	0,17
Estrone (E1)	3	2	4	5	1,67	0,83

All other steroids had samples with concentrations bellow LOQ. For statistical purposes we decided to use < LOQ concentrations for 11-deoxyCOR and DOC steroids, since those were comparable with > LOQ concentrations and provided a better overall data distribution, as recommended by Keizer et al. (2015). Since < LOQ concentrations were below zero for remaining steroids, the LOQ/2 values for statistical analyses were used for these remaining steroids (Table 2.4).

2.2.7. Behavioral study methods during suckling and post-weaning fast periods

Behavior of grey seal pups, located on the Isle of May, Scotland (56◦11'N, 02◦33'W) was observed during the breeding season (October-December) of 2017. A specific location on the island – Tarbet Hole – was chosen for the observation. Less than a hundred females congregate at the same time during the peak of a breeding season and a similar number of pups are born. There are also 3-7 dominant males resting in the area at different times, guarding groups of 7-12 females with pups. On one side the haul out is surrounded by a high cliff and is isolated from other grey seal haul outs which limits the migration of animals. A higher observation point from the cliff allows simultaneous observations of several dozen pairs of females and pups without disturbing them (Fig. 2.2).

For the clarity purposes, observations were performed in two years (2013 and 2017) during the breeding season of grey seals on the Isle of May. Samples were collected together with behavioral observations from same animals, however samples from 2013 were used for optimizing gas chromatography tandem mass spectrometry method (GS-MS/MS). Despite many efforts, it was impossible to optimize the instrument with confidence and results of steroid concentrations thus could not be used. Therefore no data from that year will be included in this work. This situation is related to the small sample size for a link between hormones and behavior.

Suckling period. Behavioral recordings took place during daylight hours from 8 AM to 18 PM. Individuals were observed for an average of 2 hours per day at one location.

Although a pilot study of nocturnal grey seal behavior by (Culloch et al., 2016) showed some differences in diurnal and nocturnal behavioral budgets in females and pups during the breeding season, most grey seal observations take place during the day (Bishop et al., 2015b; Bowen, 1991; Kovacs, 1987b;

Lydersen and Kovacs, 1999), because observations are particularly difficult to make in the dark. However, the aim of this study is to assess differences in behavioral budgeting, so it is important to monitor behavior when its greatest diversity occurs, which is during daylight hours (Culloch et al., 2016).

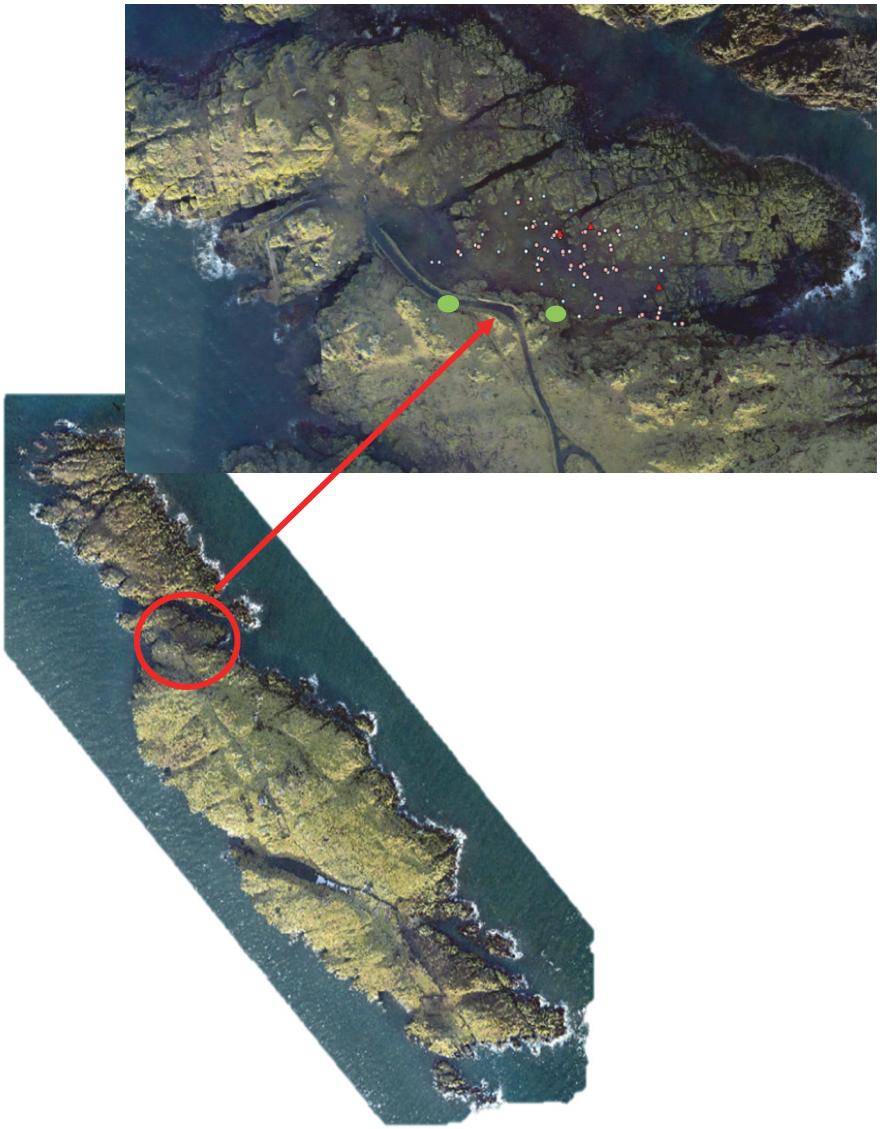


Fig. 2.2. A high resolution stitched image of Isle of May (bellow) and Tarbet hole (above) with mum (pink circles), pup (white circles) locations and adult male (red triangles) locations. Green circles indicate locations observations were taken from.

For the comparative analysis behavioral data from grey seal pups, included in other SMRU studies (sampled for blood and saliva samples, body mass, etc.) and suitable for their location – well visible from the observation points (Fig. 2.2) was included. Behavioral recording began when observers arrived at the observation site. The scan sampling method was chosen for behavioral observations. This method captures the states of individuals in recurring time periods in an effort to capture change as quickly as possible (Altmann, 1974). In this study, pup behavioral states (see ethogram, Table 2.5) were recorded every 5 minutes. The scan sampling method is suitable for assessing the behavior of several individuals at once, thus saving time and resources. In this method, only states of behavior lasting more than a few seconds can be estimated, and it is not statistically possible to estimate absolute time in the evaluation, but only to deduce the frequency and proportion of behavior (Altmann, 1974). Up to 11 individuals were observed simultaneously.

During scan sampling was recorded:

1) Time of the day.
2) Pup ID.
3) Behavioral states (see ethogram, Table 2.5).
4) Another individuals age/ sex/ other ID (if possible) involved in the interaction if the element of pup's behavior was of social nature.

Table 2.5. Ethogram for grey seal pup behavioral analysis.

Period	Abbreviation	Short description	Long description
Suckling	MP	*Mother pup interaction*	Physical contact between the pup and its mother. The concept of contact may include mother or pup touching each other by the mouth or flipper, a gentle bite, climbing or other social contact other than breastfeeding.
Suckling	Skl	*Suckle*	Breastfeeding.
Both	Loc	*Locomotion*	Movement to any direction.
Both	A	*Alert*	A state of alertness in which the body of an individual is tense, the neck is outstretched, and the head is raised; the individual checks the environment (looks around, smells the air and listens).

Period	Abbreviation	Short description	Long description
Both	SI - D	*Social investigation per distance*	One individual looks tentatively at another individual with his head held slightly low, smells another individual from a distance, or shows other signs of interest in another individual. No contact.
Post-weaning fast	SI - C	*Social investigation per contact*	One individual sniffs another individual or shows other signs of interest, touching a mustache, fins, snout, bite. Physical contact of individuals is visible. When physical contact between the pup and the mother is observed, it is marked MP, no other social investigation per contact was recorded between a pup and another individual.
Both	NI	*Non-social investigation*	Exploring the environment by contact with inanimate objects using a mustache, snout, mouth, or anterior fins.
Both	SP	*Social play*	Any social play between young individuals (suckling and weaned pups), including play fight and imitation of mating.
Both	NP	*Non-social play*	An non-social play directed at inanimate objects (stones, grass, etc.), expressed by grabbing, pulling, and throwing them; is also defined by repetitive, exaggerated, jerking, or distorting movements of the pup's body.
Both	Ag	*Agonistic behavior*	Any agonistic behavior, directed at another individual; raised whiskers and open mouth threat, sometimes accompanied by vocalizations, sudden tilting toward another individual, aggressive biting.
Both	R	*Rest*	Period of inactivity, including sleep and comfort moves.
Both	Voc	*Vocalization*	Vocalization - an individual with an open mouth, emits sound.

Only half of the observation colony could be seen from one observation site, so the observation time was divided between the two locations randomly (Fig. 2.2). While behavior in one location was monitored and recorded, the behavior of pups in another location was captured by a built-in high-definition video cam-

era (Panasonic HDC-TM60 HD 1920 × 1080). Recordings were later analyzed using the Solomon Coder (Version: beta 17.03.22, András Péter, 2017) computerized behavior recording program. Behavioral data from field datasheets and Solomon coder program was transferred to MS Excel program for further processing.

Suckling pups were identified on the basis of maternal identification and external individual marking marks. During the suckling period, especially at the beginning, females rarely withdraw from their pups, so the identity of the pups is identified on the basis of the mother's identity. The identity of the mother is determined on the basis of external marking methods (codes of letter-number combinations burned out many years ago, plastic markers in the flippers and the individual coat spot characteristic of grey seals. However, when females were not around, white pups were hard to discriminate and recognize among each other even with plastic tags attached. To increase the accuracy of identification, target pups were marked by gluing patches of black material of different shapes (circle, square, triangle, rectangle) with a diameter of 5 cm during capture. Each pup had a marking of the particular shape that helped to recognize it. Once pups were almost fully molted (stage 4 according to Kovacs and Lavigne (1986)), it became possible to identify them according to their pelage markings.

During the entire observation period from 25 October to 29 November 2017. The behavior of a total of 27 pups was recorded, 16 of which were subsequently selected for further analysis, because they had number of scans recommended as suitable for behavioral analysis (n = 150) that would cover approximately two – three days of daylight (Bishop et al., 2017). However, only 5 individuals observed during the suckling period (Table 2.6) were sampled for steroid analysis and were included in further data analysis.

Post-weaning fast. Individual behavior of weaned grey seal pups was recorded during daylight hours from 8 AM until 4 PM local time in a period from the 14 November to 9 December using the same high-definition video camera (Panasonic HDC-TM60 HD 1920 × 1080) as during the suckling period. Location of the target animals was chosen ad libitum, depending on animal's ID and number of target individuals. A focus was made on aggregations of seals (more than one individual) to investigate social behavior patterns during the post-weaning fast and also if more than one target individual could be seen in a frame and increase the amount of behavioral data obtained from one video file. Once the aggregation of the pups with a target individual was found, the recording camera was positioned so that it would not to disturb the individuals.

One location was filmed for one to six hours per day, depending on how many subjects were seen in the frame. If there were less than one subject in the frame, after 2-3 hours of filming one aggregation, another aggregation was searched for and recording continued.

To help quickly localize the target animal in the surrounding area, target pups were marked with rhodamine paint. Markings were carried out in such a way that caused the least possible disturbance to the pup itself and to the surrounding individuals in the colony. A sponge soaked with a pink rhodamine paint was attached to the end of a 1,5-meter-long pole, lowered against the pup, and used to mark with an individual combination of lines and dots at a specific area of the body different for each pup. Rhodamine was chosen as a dye because of its non-irritating odor, as grey seals have a sensitive smell. In addition, rhodamine is lightly fluorescent in the twilight, easily visible from a distance and washes away with water over time.

For this particular study during the post-weaning fast the results from visual material of 5 individuals were included in analysis (2 females and 3 males, Table 2.6). Individuals were observed at 31 ± 4 (mean ± SD) days of age for about 0,96 ± 0,12 (mean ± SD) hours per day and 5,05 ± 1,14 (mean ± SD) hours totally.

Table 2.6. 6 pups (5 pups during suckling and post-weaning fast, however only 4 were observed during both periods) were involved in both – physiological (blood and saliva) and behavioral study – 2 females, 4 males. *eq*. – equivalent to hours/ scans.

Pup ID	**Pup sex**	**Nutritional period**	**Mean body mass (kg)**	**Mean age (days)**	**No. of days observed**	**Days of age covered (mean ± SD)**	**No. of hours covered**	**No. of scans covered**
58038	M	Suckling	39,90	11	14	8 ± 5	eq. 20,83	250
N6J	M	Suckling	25,80	8	16	9 ± 5	eq. 30,92	371
OJ	F	Suckling	27,40	11	15	9 ± 5	eq. 22,67	272
74790	F	Suckling	24,40	13	10	6 ± 3	eq. 21,42	257
74890	M	Suckling	24,40	9	10	10 ± 5	eq. 16,08	193
58038	M	Post-weaning	41,80	32	6	34 ± 6	6,14	eq. 74
N6J	M	Post-weaning	51,40	29	4	33 ± 2	3,62	eq. 44
OJ	F	Post-weaning	30,70	31	6	28 ± 8	6,14	eq. 82
74790	M	Post-weaning	32,40	31	5	33 ± 4	5,19	eq. 62
75334	F	Post-weaning	46,80	32	4	26 ± 1	4,18	eq. 50

2.2.8. Statistical analysis

Statistical data analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and statistical differences considered to be significant when $p < 0.05$. Further details on statistical analyses are presented in sections bellow.

2.2.8.1. ELISA validation

Inter-assay CV of plasma samples was estimated using two samples from 2012, each measured in duplicate on each of the two plates (Plate 1 (analyzed 2014), Plate 2 (analyzed 2015)). Mean and standard deviation concentrations were calculated for each sample in each plates and the standard deviation expressed as a percentage of the mean to give inter-assay CV. The average inter-assay CV of the two samples is reported here. All saliva samples were measured within one plate; thus only intra-assay CV is reported. Intra-assay CV was calculated from the mean and standard deviation of the duplicates of each sample in the same plate and the mean intra-assay CV calculated.

Parallelism was determined by visually investigating the graphs in which optical density of standard curve and pooled saliva and plasma samples were plotted against dilution. Accuracy of plasma and saliva kits was determined by performing linear regression of diluted samples (plasma: $n = 6$; saliva: CS, TS $n = 6$, ES $= 5$) of two plates comparing observed versus expected measurements, which should show a positive linear relationship with a slope of approximately 1.0 (a slope of 0.8 – 1.2 and $r^2 > 0.95$ was considered acceptable) (Hunt et al., 2014; Kershaw and Hall, 2016). Expected and observed concentrations in high and low spiked saliva and plasma samples were compared using a paired t-test.

Dilution values were considered acceptable if they were within 30 % of the expected concentrations for low concentrations, and recoveries of spiked samples if they were within 20 % of expected values in both saliva and plasma (Yan, 2018). Precision of intra- and inter-assay CV was considered acceptable when < 20 %.

2.2.8.2. Comparability of steroid concentrations in saliva and plasma

Linear regression analysis was used for determining the relationship between cortisol concentrations in saliva and plasma. Nonparametric Spearman rank correlation was used to compare estradiol and testosterone concentrations in saliva and plasma. Results were presented as overall or

partial showing relationship between saliva and different ELISA plates for analysis (Plate 1 (collected in 2012, analyzed in 2014), Plate 2 (collected in 2012, analyzed in 2015), Plate 4 (collected in 2017, analyzed in 2018)).

Bland-Altman analysis was used to explore the agreement between steroid measurements in saliva and plasma from the same animal on the same occasion (Bland and Altman, 2007; Giavarina, 2015). Absolute mean differences were calculated by subtracting each sample plasma value from its corresponding matched saliva value (e.g. ES-EB) and proportional difference ratio was calculated by dividing absolute difference from the mean of two methods (e.g. (ES-EB)/((ES+EB)/2) * 100 %). An agreement interval of difference ratio, within which 95 % of the differences of the saliva measures from plasma lay, was calculated by adding or subtracting 1.96 x SD of difference ratio from the mean measures (Mean ratio ± 1.96SD). Results were presented as overall or partial showing relationship between saliva and different ELISA plates for analysis (Plate 1 (collected in 2012, analyzed in 2014), Plate 2 (collected in 2012, analyzed in 2015), Plate 4 (collected in 2017)).

2.2.8.3. Saliva steroid concentration dependence on sample volume

The ELISA manufacturer recommends using saliva samples of > 0,5 ml of volume for the analysis. However, the required saliva sample volume was not always obtained in the wild, thus Spearman rank correlation was used to evaluate whether the saliva sample volume obtained was related to steroid (ES, TS, CS) concentration. Results were given as overall correlation and partial between years of saliva collection (2012 and 2017).

2.2.8.4. Comparison of sensitivity of two matrices

A generalized linear mixed model (GLMM) was used to evaluate the possible effect of nutritional stage (S1, S2, W1, W2), nutritional period (suckling vs. post-weaning fast), pup sex, body mass (kg), age (days), and time of the day on EB and ES comparable samples (n = 27) and, additionally – plasma plate number (Plate 1, Plate 2, Plate 3) on CB and sampling year (2012, 2017) on CS comparable samples (N = 55). Only matching samples from 2012 were used for the analysis, since there was no correlation between two matrix samples from 2017. Matched saliva and plasma samples from 2012 and 2017 were used for comparison of cortisol concentrations. A normal distribution model and a linear link function was used for EB and a gamma distribution model with a log link function was used for CB, CS and ES. Scaled identity

covariance structure for nutritional stage as repeated measures for every pup (ID as a Subjects) and Satterthwaite approximation for estimating degrees of freedom was used (Zuur et al., 2009). Corrected Akaike information criteria (AICc) was used to select the best model. Residuals plots were inspected to check model fit.

2.2.8.5. Comparison of ELISA values with steroid values obtained by alternative analysis methods

Intra-class correlation (ICC) measures the extent to which different methods agree with each other and works as a reliability statistic for the measurement procedure, thus ICC two-way mixed model was used to compare concentrations of different plasma estradiol (EB_RIA vs. EB) and cortisol (CB vs. UPC_2-MS/MS) analysis methods. Consistency type of the model was used instead of absolute agreement since absolute concentrations differ between the methods. Cronbach's alpha was used to measure internal consistency ("reliability") of ICC. Concentrations between two methods were compared by using paired sample T test (t). Testosterone was excluded from analysis due to a high number of concentrations below the limit of detection (< LOD) obtained by both methods – ELISA (TB) and RIA (TB_RIA).

2.2.8.6. Behavioral analysis

An effort was made to find correlations between the proportion of grey seal pup behavior and steroid concentration from data collected in 2017 by using simple Spearman rank correlation. Mean body mass (kg) and mean age (days) was also included into comparison. Steroid concentrations used were those obtained with ELISA (ES, TS, only for the suckling period, since only 3 pups of 5 had their samples collected), RIA (EB_RIA), UPC_2-MS/MS (DHEA, AN, A5, TS, DHT, KetoTS, DOC, 11-deoxyCOR, ALDO, COS, CORNE, PREG, P4, 17α-OHP, E1). CS, CB and COR were excluded from the comparative analysis with, because of cortisol's sensitivity to the sampling duration, TB and TB_RIA were excluded due to low sample size above LOQ/ LOD. Correlation coefficient as well as level of significance (p) were compared to indicate the strongest possible correlations and thus factor for specific behavioral element. Mean proportion (%) of behavioral elements and mean concentrations of different steroids during suckling (n = 5) and post-weaning fast (n = 5) of an individual seal (n = 6). There were 4 individuals (58038, 6J, 74790, OJ) that got their behavior analyzed during both – suckling and post-weaning

fast, while remaining individuals had their behavior analyzed either during the suckling (74890) or post-weaning fast (75334) periods. Friedman's 2-way ANOVA by ranks (χ^2) was used to find significant differences between mean proportions (%) of different behaviors. Adjusted coefficient of significance (Dunn's test) was used for multiple pairwise comparisons.

2.3. RESULTS

2.3.1. Success of sample collection

From a total of 102 plasma samples collected (2012 – 71, 2017 – 31) when an attempt to collect saliva as well was made (no saliva samples were collected in 2016), 12 samples were used for ELISA optimization (more information on sample use in the Supplementary Material Table S2), thus 91 (2012 – 60, 2017 – 31) plasma samples were used for validation and further statistical analysis. We successfully collected 62 (2012 – 38, 2017 – 24) saliva samples, which represents 60,19 % (2012 – 54 %, 2017 – 77 %) of all 102 sampling occasions. Unsuccessful saliva sampling events (n = 40) were due to limited saliva production, which did not provide the minimum amount of saliva during anesthesia, or because samples were contaminated with blood, and therefore had to be discarded during the collection procedure. Even though the manufacturer recommends the collection of at least 0,5 ml of sample volume, saliva analysis of all three steroids using our given commercial kits requires a minimum of 250-300 µl (TS and CS – 50 µl and ES 25 µl for each well). Where partial samples were obtained (Supplementary material Fig. S1), containing less than the minimum recommended for the assays, the volume obtained was used for one or two steroid analyses, as permitted, and prioritized as described earlier. From samples that were collected, 9 (14,52 %) were partial samples (six samples when only two steroids and four samples when only one steroid was analyzed) and 53 (84,48 %) full saliva samples available. Partial samples were more likely to be collected when animals were more alert and mobile during anesthesia (pers. obs.). Therefore, enough saliva to analyze all three steroids was obtained in ~52 % of all the animal sampling events. There were also three samples (two from nutritional stage S1 and one from S2) of TS collected in 2012 that were above the limit of detection, and there was not enough sufficient sample or reagent to perform a subsequent dilution. These high samples were not included in further statistical analysis (more detail in Table S2).

2.3.2. Validation of ELISA kits for different matrices

2.3.2.1. Plasma (EDTA) validation

Plates for EB and CB were validated successfully, as they showed intra-CV < 15 % and spike recovery rates within 20 % of expected (Table 2.7). Expected vs. observed spiked concentrations were not different for both

steroids (CB (n = 4): t = 0,24, df =3, p = 0,83, EB (n = 6): t = -0,24, df = 5, p = 0,82). Dilution curves were parallel to standard curves for CB and EB (Fig. 2.3 and Fig. 2.4). Inter-assay CV was 8 % for EB and 25 % for CB. Expected vs. observed concentrations of diluted samples showed a linear relationship with a slope close to 1 for both CB (r^2 = 0,95, df = 5, p < 0,01, slope – 0,95) and EB (r^2 = 0,97, df = 5, p < 0,001, slope – 0,92).

TB intra-assay CV was < 11 % (except 23 % from a Plate 4, most likely due to very low concentrations) and an average spike recovery was 106 % (Table 2.7). Expected vs. observed spiked TB concentrations were not significantly different (n = 6, t = -0,642, df = 3, p = 0,57). Dilution curves were parallel to the standard curve (Fig. 2.5) and dilution performance was good with a slope close to 1 (r^2 = 0,99, df = 5, p < 0,001, slope – 0,99). However, inter-assay CV was unacceptably high (86 %), thus this ELISA for plasma testosterone was not successfully validated.

Concentrations of steroid hormones were overall CB > TB > EB, however absolute mean values differed between the plates within a steroid (Table 2.7).

2.3.2.2. Saliva validation

Dilution curves were parallel to standard curves for all steroids in saliva (Fig. 2.3-2.5). All three kits were successfully validated. Intra-assay CV for all steroids was < 11 %. Spike recovery rates were within 20 % of expected for TS and CS, and within 30 % for ES (Table 2.8). Expected vs. observed spiked concentrations were not different for all three steroids (CS (n = 4): t = 0,76, df =3, p = 0,5, ES (n = 4): t = -1,09, df = 3, p = 0,36, TS (n = 4): t = 0,01, df = 3, p = 0,99). Expected vs. observed concentrations of diluted samples showed a linear relationship with a slope close to 1 for all – saliva cortisol (r^2 = 93, df = 5, p < 0,01, slope – 0,96), estradiol (r^2 = 0,88, df = 4, p = 0,015, slope – 0,94) and testosterone (r^2 = 0,98, df = 5, p < 0,001, slope – 0,99).

Saliva steroid hormone concentrations reflected the concentration pattern found in blood: CS > TS > ES (Table 2.8). All saliva steroid concentrations differed significantly between different years. Average ES concentration was about 40 % lower (n = 53, MW U = 480, p = 0,012) in 2012 (11,77 ± 8,29 pg/ml) samples than in 2017 (15,35 ± 1,51 pg/ml). In comparison, TS was about 55 % higher (n = 56, MW U = 224, p < 0,01) in 2012 (131,37 ± 82,91 pg/ml) than in 2017 (86,14 ± 83,33 pg/ml) and average CS concentration was around 57 % higher (n = 60, MW U = 168, p < 0,001) in 2012 (0,21 ± 0,02 μg/dl) than in 2017 (0,12 ± 0,01 μg/dl).

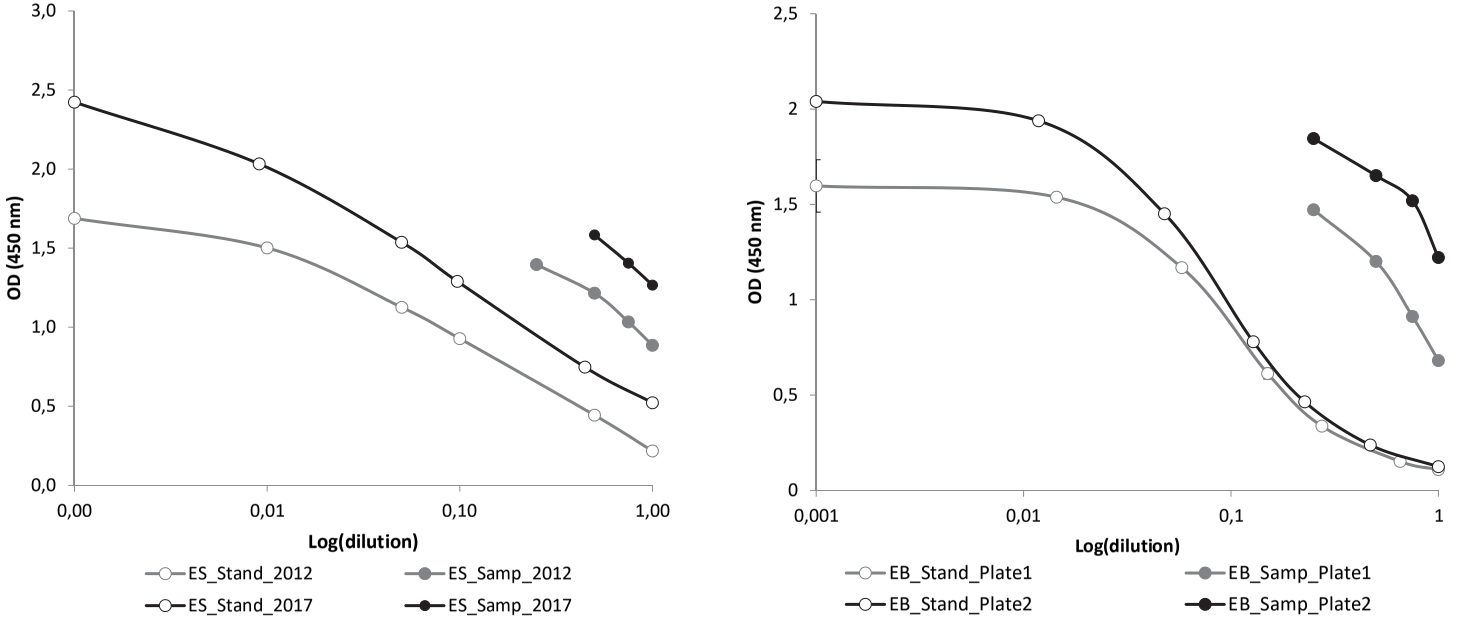


Fig. 2.3. Parallel curves of diluted pooled samples of estradiol in saliva (ES) and plasma (EB) to their standard curves. Plasma standard and sample dilution curves are provided for each plate. ES_Stand – ES standard curve, ES_Samp – ES dilution curve of pooled saliva sample, EB_Stand_Plate1/Plate2 – EB standard curve of Plate 1 or Plate2, EB_Samp_Plate1/Plate2 – EB dilution curve of pooled plasma sample in Plate1 or Plate2. Pooled plasma samples are from different samples for Plate1 and Plate2.

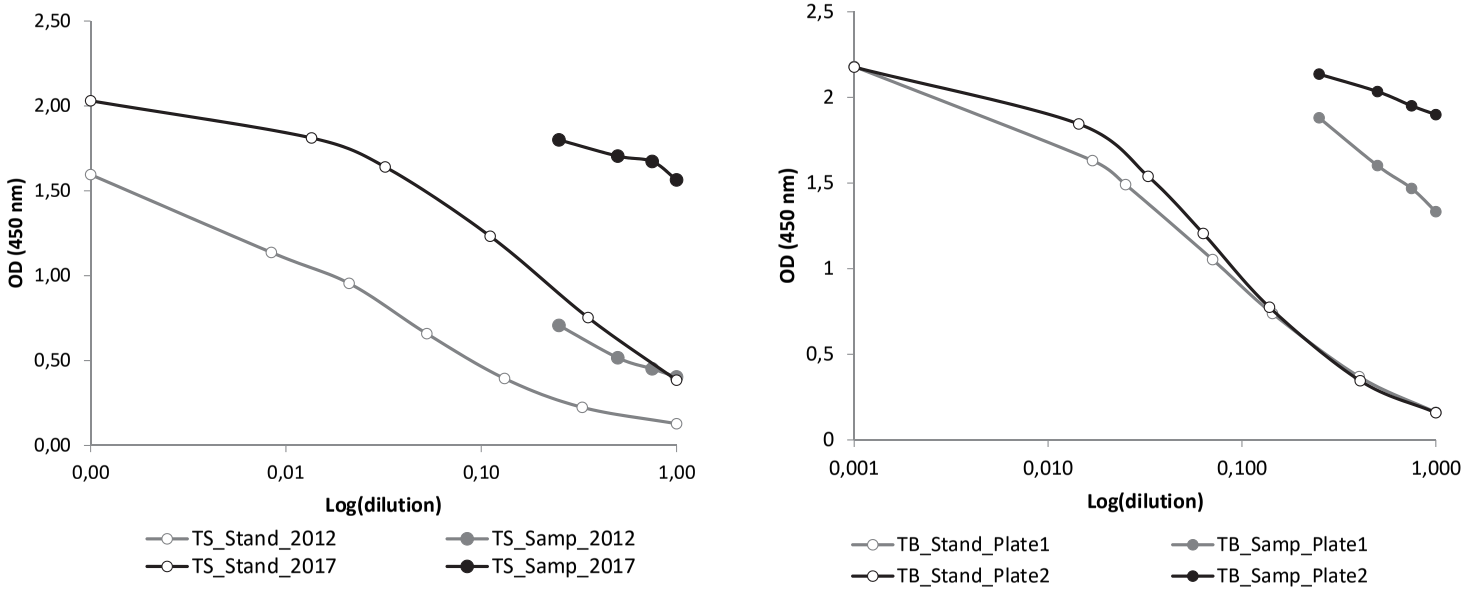


Fig. 2.4. Parallel curves of diluted pooled samples for testosterone in saliva (TS) and plasma (TB) to their standard curves. Plasma standard and sample dilution curves are provided for each plate. TS_Stand – TS standard curve, TS_Samp – TS dilution curve of pooled saliva sample, TB_Stand_Plate1/Plate2 – TB standard curve of Plate 1 or Plate2, TB_Samp_Plate1/Plate2 – TB dilution curve of pooled plasma sample in Plate1 or Plate2. Pooled plasma samples are from different samples for Plate1 and Plate2.

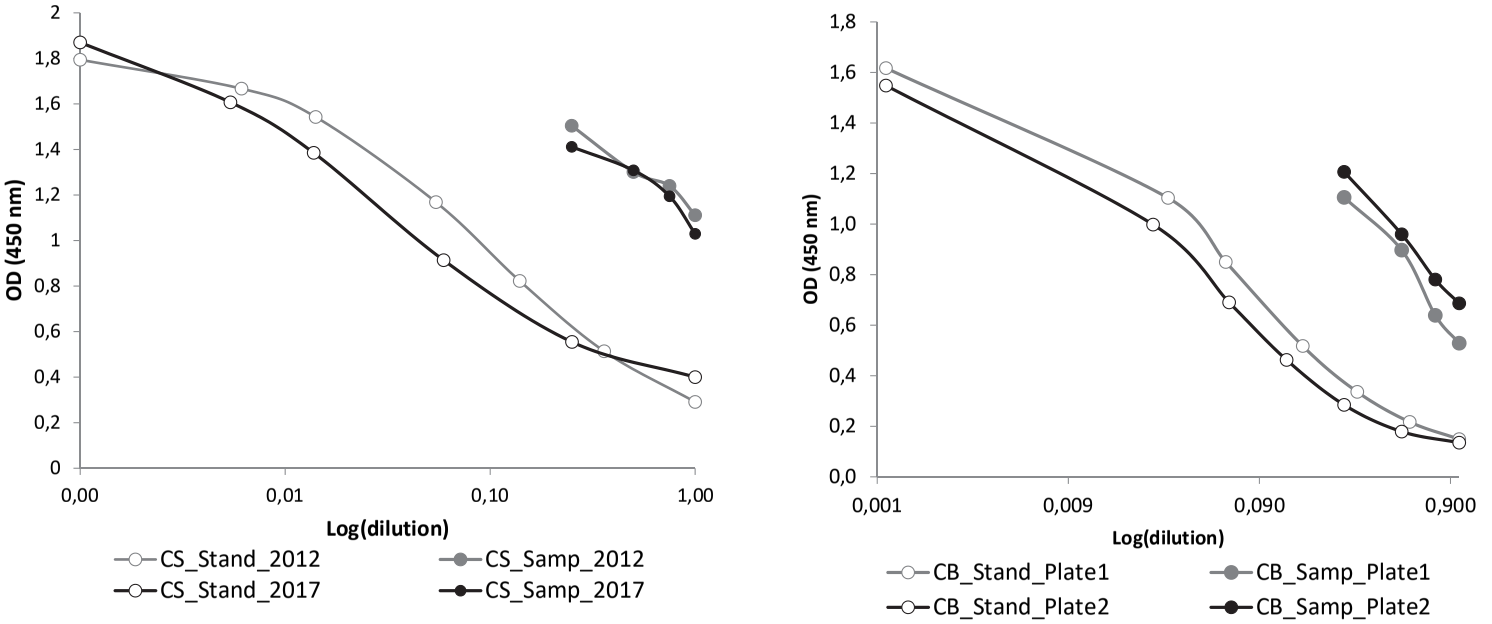


Fig. 2.5. Parallel curves of diluted pooled samples for cortisol in saliva (CS) and plasma (CB) to their standard curves. Plasma standard and sample dilution curves are provided for each plate. CS_Stand – CS standard curve, CS_Samp – CS dilution curve of pooled saliva sample, CB_Stand_Plate1/Plate2 – CB standard curve of Plate 1 or Plate2, CB_Samp_Plate1/Plate2 – CB dilution curve of pooled plasma sample in Plate1 or Plate2. Pooled plasma samples are from different samples for Plate1 and Plate2.

Table 2.7. Descriptive statistics of ELISA analysis of three steroid hormones – testosterone (TB), estradiol (EB), cortisol (CB) and dihydrotestosterone (DHT) – measured in plasma from suckling and fasting grey seal pups collected in different years and analyzed within different plates (2012 – Plate 1 and Plate 2, 2016 – Plate 3, 2017 – Plate 4).

Hormone	**Plasma testosterone (TB), ng/ml**				**Plasma estradiol (EB) pg/ml**				**Plasma cortisol, ng/ml**		
Plate Nr. (Year of collection)	Plate 1 (2012)	Plate 2 (2012)	Plate 3 (2016)	Plate 4 (2017)	Plate 1 (2012)	Plate 2 (2012)	Plate 3 (2016)	Plate 4 (2017)	Plate 1 (2012)	Plate 2 (2012)	Plate 4 (2017)
Mean ± SE	0,7 ± 1,12	0,2 ± 0,24	0,4 ± 0,46	0,1 ± 0,09	233,45 ± 72,29	96,48 ± 64,31	33,64 ± 30,2	11,68 ± 10,14	107,62 ± 27,57	49,4 ± 22,25	48,58 ± 15,96
Min – Max	0,51 – 1,12	0,05 – 1,26	0,06 – 0,96	0,01 – 0,39	150,43 – 385,9	16,7 – 236,88	1,85 – 148,3	0,64 – 40,17	63,97 – 161,25	16,56 – 99,98	22,21 – 87,2
N total	33	28	30	22	33	28	30	32	33	28	32
N < LOD	-	-	24	13	-	-	-	1	-	-	-
Obtained/expected % of sample dilution:											
1 is the measured concentration of pooled sample	1 = 0,56	1 = 0,18	-	-	1 = 224,47	1 = 141,49	-	-	1 = 105,43	1 = 49,43	-
0,75 is 75% sample mix	95,63 %	105,84 %	-	-	95,05 %	82,13 %	-	-	97,45 %	102,10 %	-
0,5 is 50 % sample mix	99,55 %	87,57 %	-	-	83,18 %	95,07 %	-	-	73,05 %	92,59 %	-
0,25 is 25 % sample mix	72 %	72,32 %	-	-	71,23 %	109,79 %	-	-	81,87 %	81,49 %	-
RR % low spiked	115,58 %	91,30 %	71,68 %	-	89 %	119,37 %	109,85 %	-	104,68 %	100,02 %	-
RR % high spiked	122,56 %	92,61 %	83,29 %	-	94,14 %	99,26 %	91,51 %	-	103,8 %	90,69 %	-
Intra-assay CV (%)	8,54 %	11,04 %	5,2 %	21,13 %	5,15 %	2,84 %	1,64 %	13,18	6,35 %	3,93 %	6,73 %
Inter-assay CV (%)	86,24 % (n = 2)		-	-	8,49 % (n = 2) -		-	-	25,22 % (n = 2)		-

Table 2.8. Descriptive statistics of ELISA analysis of three steroid hormones – testosterone (TS), estradiol (ES) and cortisol (CS) – measured in saliva from suckling and fasting grey seal pups collected in different years (2012 and 2017).

Hormone	**Saliva testosterone (TS, pg/ml)**		**Saliva estradiol (ES, pg/ml)**		**Saliva cortisol (CS, µg/dl)**	
Year of analysis	2012	2017	2012	2017	2012	2017
Mean ± SD	131,37 ± 82,91	86,14 ± 83,33	11,77 ± 8,29	15,35 ± 7,07	0,212 ± 0,1	0,12 ± 0,05
Min – Max	31,07 – 313,42	18,75 – 396,88	4,45 – 42,45	4,34 – 34,24	0,065 – 0,479	0,05 – 0,25
n	32	24	31	22	37	23
n < LOD	-	-	-	-	-	-
Obtained/expected % of sample dilution:						
1 is the measured concentration of pooled sample	1 = 111,80	1 = 37,45	1 = 11,61	1 = 9,8	1 = 0,25	1 = 0,12
0,75 is 75% sample mix	100,69 %	91,44 %	83,97 %	101,59 %	92,31 %	81,23 %
0,5 is 50 % sample mix	116,63 %	114,25 %	66,17 %	92,94 %	114,62 %	85,71 %
0,25 is 25 % sample mix	118,36 %	133,88 %	59,28 %	(sample was < LOD)	106,15 %	121,01 %
RR % low spiked	119,74 %	100,28 %	104,59 %	67,85 %	91,97 %	110, 41 %
RR % high spiked	101,57 %	95,90 %	76,13 %	124,03 %	98,21 %	114,2 %
Intra-assay CV (%)	3,89 %	11,21 %	4,45 %	5,09 %	8,35 %	9,43 %
Inter-assay CV (%)	N/A	N/A	N/A	N/A	N/A	N/A

2.3.3. Sample volume and time of the day effect on saliva steroid concentration

There was no relationship between time of the day when sample was collected and steroid concentrations in any of the saliva samples (ES: n = 53, TS: n = 56, CS: n = 59, Spearman, $p > 0,05$).

The saliva sample volume showed a significant negative overall correlation with ES (n = 53, r = -0,41, $p < 0,01$) (Fig. 2.6) and TS (n = 56, r = -0,52, $p < 0,001$) (Fig. 2.7) and, but not with CS (n = 60, r = -0,02, p = 0,91) (Fig. 2.8). However, correlation differed between the years. Moderate negative correlation with TS was found in 2012 (n = 32, r = -0,35, p = 0,047) and good in 2017 (n = 24, r = -0,645, $p < 0,01$), while there were no significant correlations between saliva volume and ES in 2012 (n = 31, r = -0,32, p = 0,08), but a strong significant negative correlation was found in 2017 (n = 22, r = -726, $p < 0,001$).

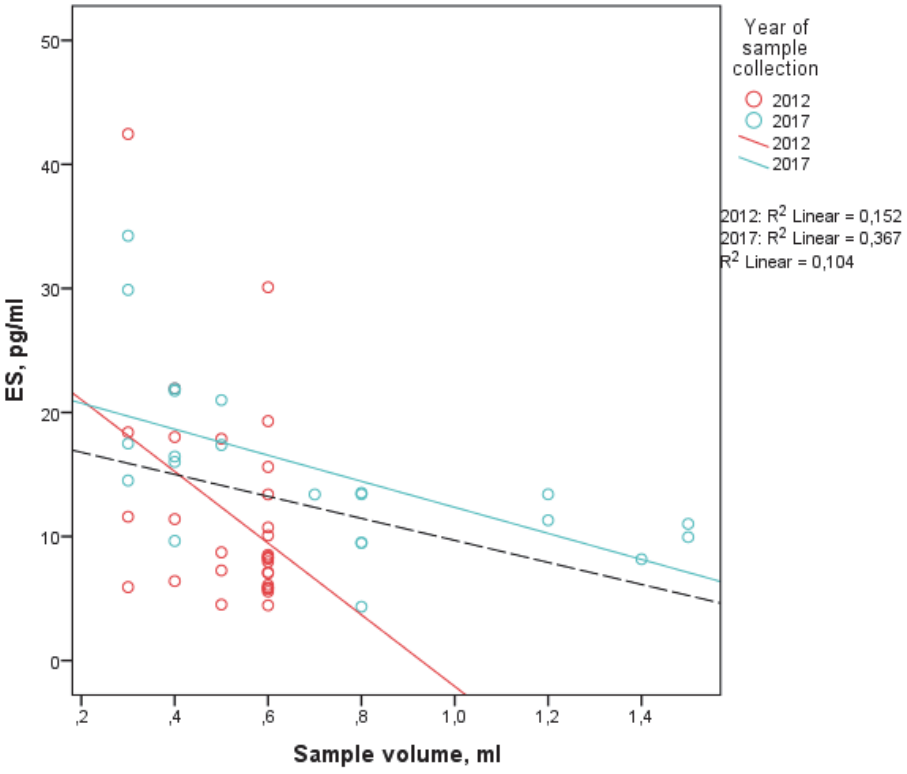


Fig. 2.6. ES concentrations (pg/ml) in different saliva volume (ml) in different year. Black dashed line indicates an overall linear regression.

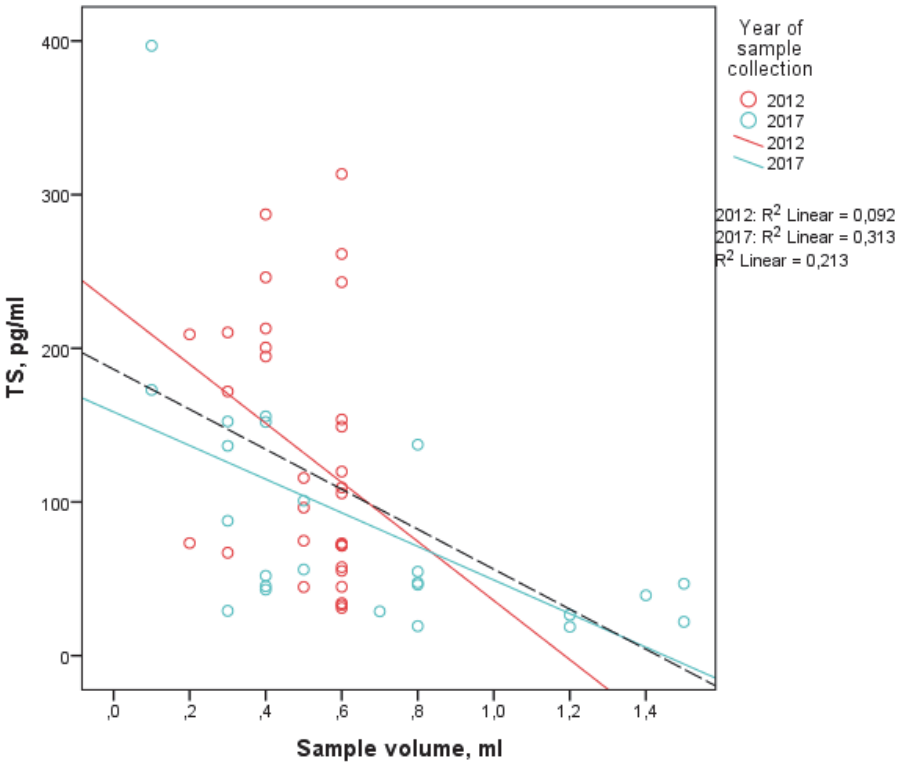


Fig. 2.7. TS concentrations (pg/ml) in different saliva volume (ml) in different year. Black dashed line indicates an overall linear regression.

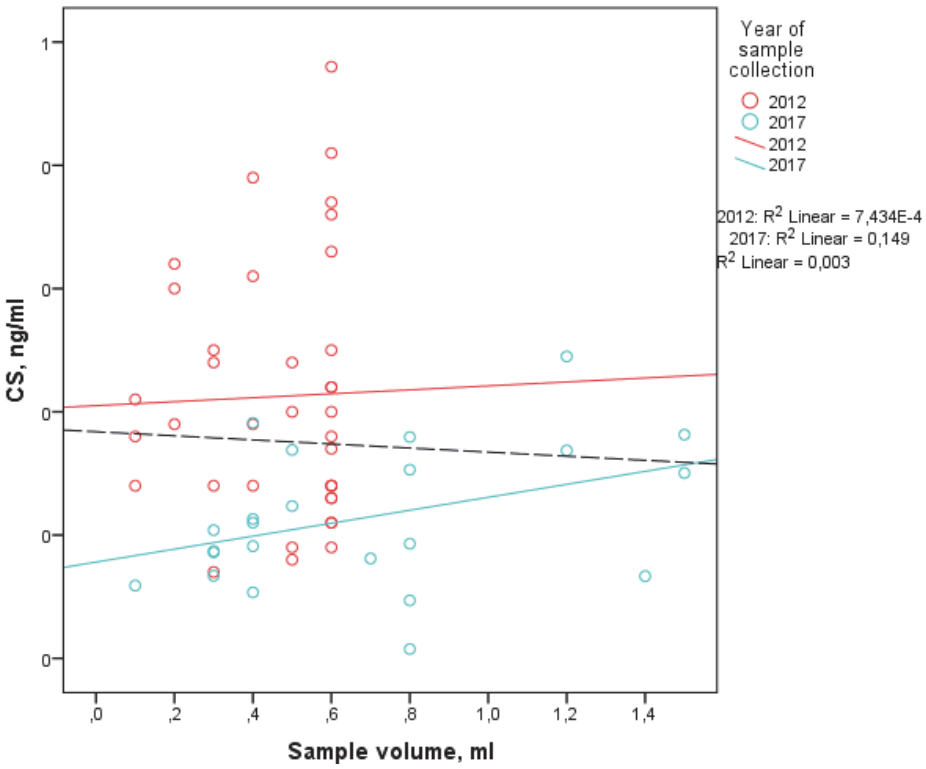


Fig. 2.8. CS concentrations (ng/ml) in different saliva volume (ml) in different year. Black dashed line indicates an overall linear regression.

2.3.4. Correlations between steroid concentrations in saliva and plasma

It was possible to compare the relationship between 55 cortisol, 49 estradiol and 44 testosterone samples in the two matrices, with a fewer matched samples for E and T due to limited saliva sample volume. Values of TS in three samples from 2014-year analysis were above the standard curve limit (760 pg/ml) and there was not enough sample to repeat the assay with a dilution, therefore they were not included in the analysis. Also, a substantial amount of plasma samples from 2017 were below the limit of detection, thus only a small amount of matched samples could be used for correlation.

There was no significant overall relationship between ES and EB concentrations (N = 49, Spearman R = -0,09, p = 0,54) (Fig. 2.9). However, the relationship differed between the plates: the concentrations in Plate 1 showed a stronger relationship (Plate 1: N = 20, r = 0,61, p < 0,01), but no

relationship was observed with plasma concentrations from remaining plates (Plate 2: N = 7, r = 0,04, p = 94; Plate 4: N = 22, r = 0,13, p = 0,56), which had lower plasma estradiol concentrations.

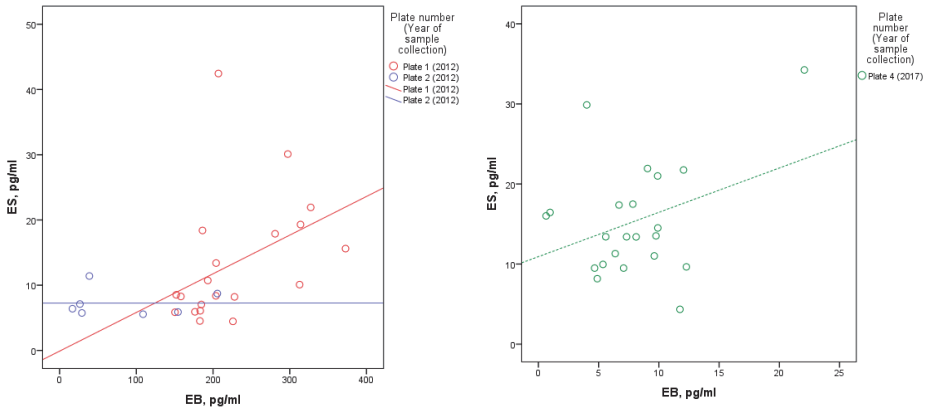


Fig. 2.9. Relationship between ES and EB concentrations between different plasma ELISA plates and years of sample collection.

There was a good significant overall relationship between CB and CS (N = 55, r = 0,63, r_{adj}^2 = 0,4, $F_{1,53}$ = 34,66, p < 0,001). Cortisol concentrations showed a strong correlation between saliva and plasma, but only those plasma concentrations from Plate 1 and Plate 4 had a significant correlation with their respective saliva value (Plate 1: N = 23, r = 0,59, r_{adj}^2 = 0,314, $F_{1,21}$ = 11,07, p < 0,01; Plate 2: N = 10, r = 0,41, r_{adj}^2 = 0,17, $F_{1,8}$ = 1,58, p = 0,24; Plate 4: N = 22, r = 0,53, r_{adj}^2 = 0,28, $F_{1,21}$ = 7,77, p = 0,01), possibly due to lower amount of samples to compare (Fig. 2.10).

TB and TS showed no overall correlation (N = 44, Spearman r = 0,12, p = 0,44) and no significant correlations were seen within separate plates as well (Plate 1: N = 20, R = 0,13, p = 0,56; Plate 2: N = 8, r = 0,24, p = 0,57; Plate 4: N = 16, r = 0,22, p = 0,43) (Fig. 2.11).

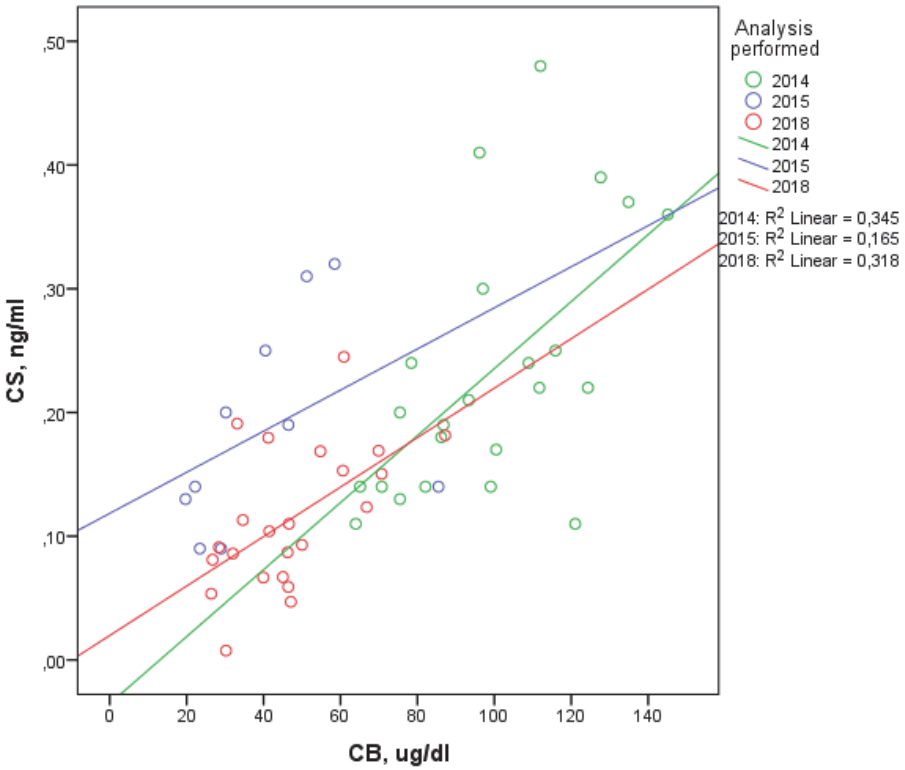


Fig. 2.10. Relationship between CS and CB concentrations from different years when analysis was performed.

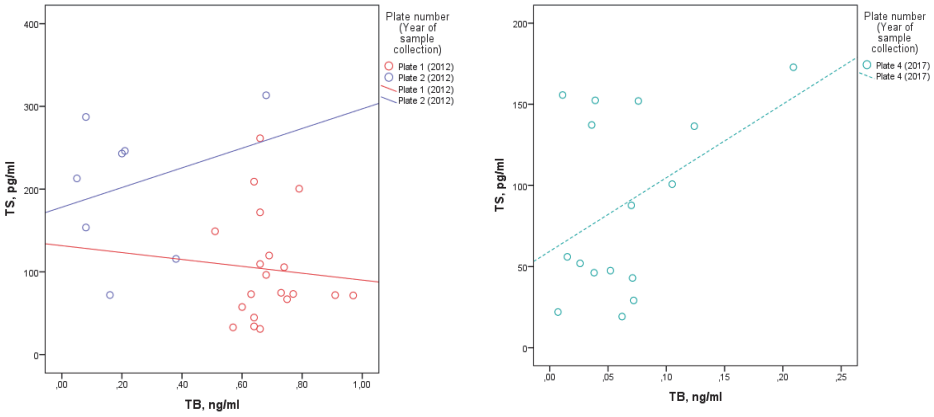


Fig. 2.11. Relationship between TS and TB concentrations between different plasma ELISA plates and years of sample collection.

2.3.5. Bland Altman difference plots

There was a very strong correlation between concentration differences of matrices and their means for all steroids (ES vs EB: r = 0,99, r_{adj}^2 = 0,98, $F_{1,47}$ = 2280,01, p < 0,001; CS vs CB: r = 1, r_{adj}^2 = 1, $F_{1,53}$ = 2518317, p < 0,001; TS vs TB: r = 1, r_{adj}^2 = 1, $F_{1,42}$ = 616164,19, p < 0,001) indicating a significant proportional bias between the two matrices (Fig. 2.12a, 2.13a, 2.14a).

The Bland Altman plots, in which proportional difference ratio is plotted against average concentration of two matrices, show that for all steroids the proportional difference is far away from the equality line (0) by up to about 200 %, but the majority of samples are within the confidence limits (Fig. 2.12b, 2.13b, 2.14b). A bigger discrepancy between the two methods at low concentrations can be observed for all steroids. However, the mean difference ratio and its confidence intervals vary between the plates in all steroids, with smallest confidence intervals, ranging just about 8 % between all plates is for cortisol (Fig. 2.13b). It shows that proportion ratio for this steroid remains stable between the plates in both matrices. However, high variation of proportional ratios between plates can be seen for estradiol (Fig. 2.12b) and testosterone (Fig. 2.14b). Specifically, estradiol shows a large saliva vs. plasma ratio variation coming from negative in the first three plates to a positive ratio, where plasma concentrations were lower than saliva, in Plate 4.

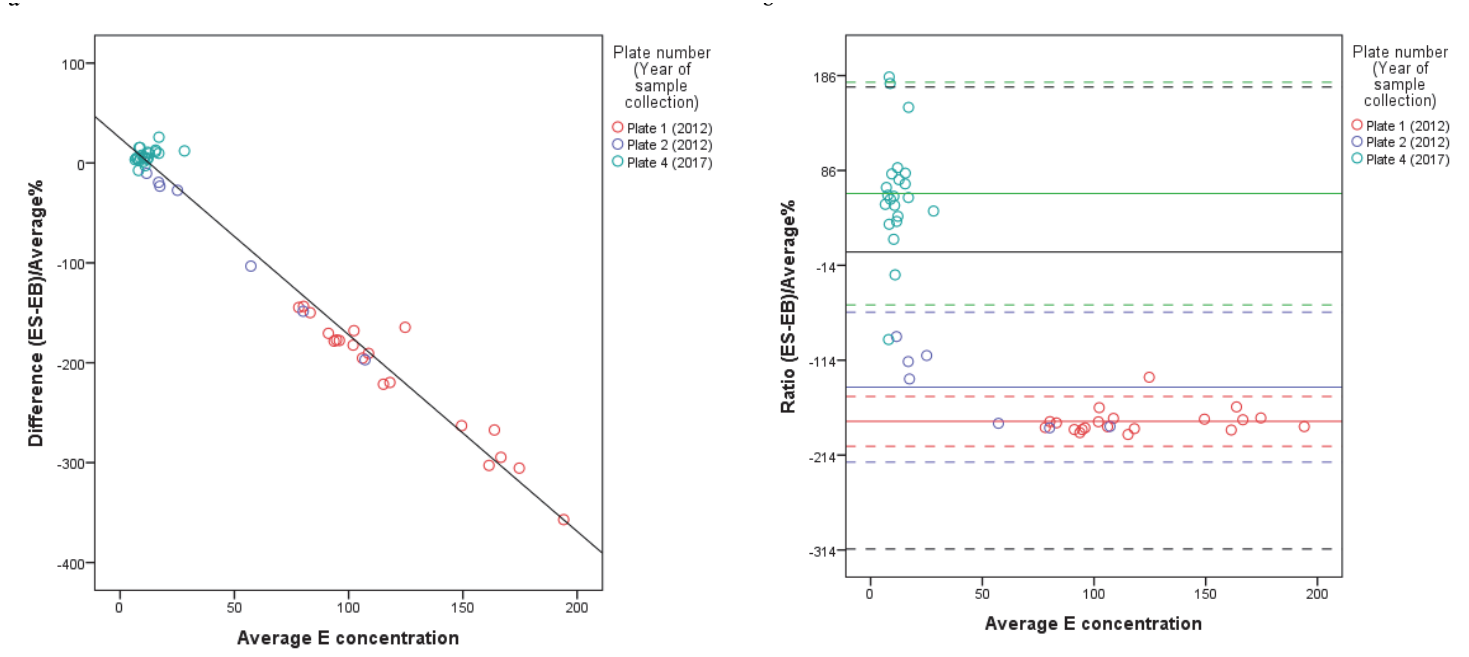


Fig. 2.12. Bland Altman plot with a) a relationship between absolute difference of estradiol (E) concentrations of two matrices and their mean concentration (different colors indicate different plates for plasma samples (Plate 1, Plate 2, Plate 4) with a linear regression line (r^2 = 0,98); b) a relationship of proportional ratio (%) of estradiol (E) concentrations of two matrices and their mean concentration (different colors indicate different plates for plasma samples (Plate 1, Plate 2; Plate 4), solid lines indicate mean bias and dashed lines indicate the 95% CI (mean +/- 1,96*SD) of mean bias).

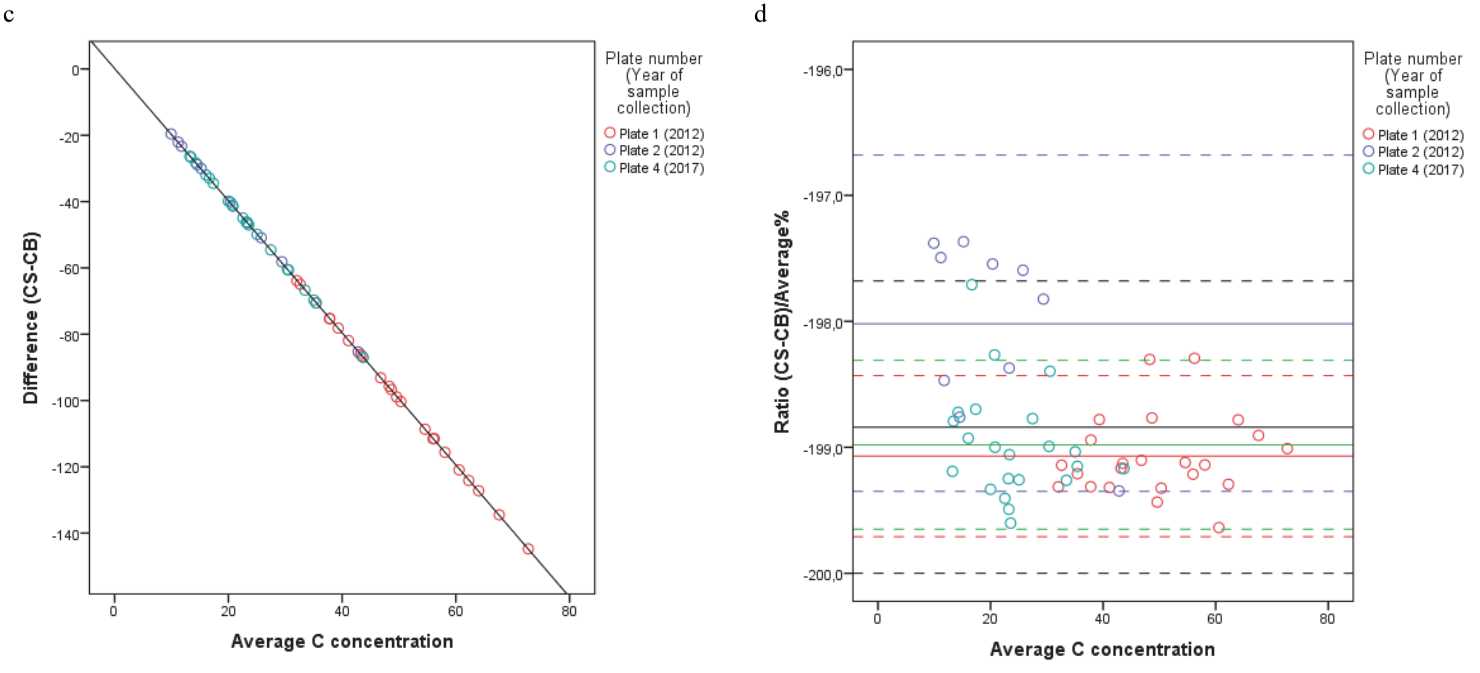


Fig. 2.13. Bland Altman plot with a) a relationship between absolute difference of cortisol (C) concentrations of two matrices and their mean concentration (different colors indicate different plates for plasma samples (Plate 1, Plate 2, Plate 4) with a linear regression line (r^2 = 1); b) a relationship of proportional ratio (%) of cortisol (C) concentrations of two matrices and their mean concentration. Different colors indicate different plates for plasma samples (black – overall, red - Plate 1, blue - Plate 2; blue - Plate 4), solid lines indicate mean bias and dashed lines indicate the 95% CI (mean +/- 1,96*SD) of mean bias).

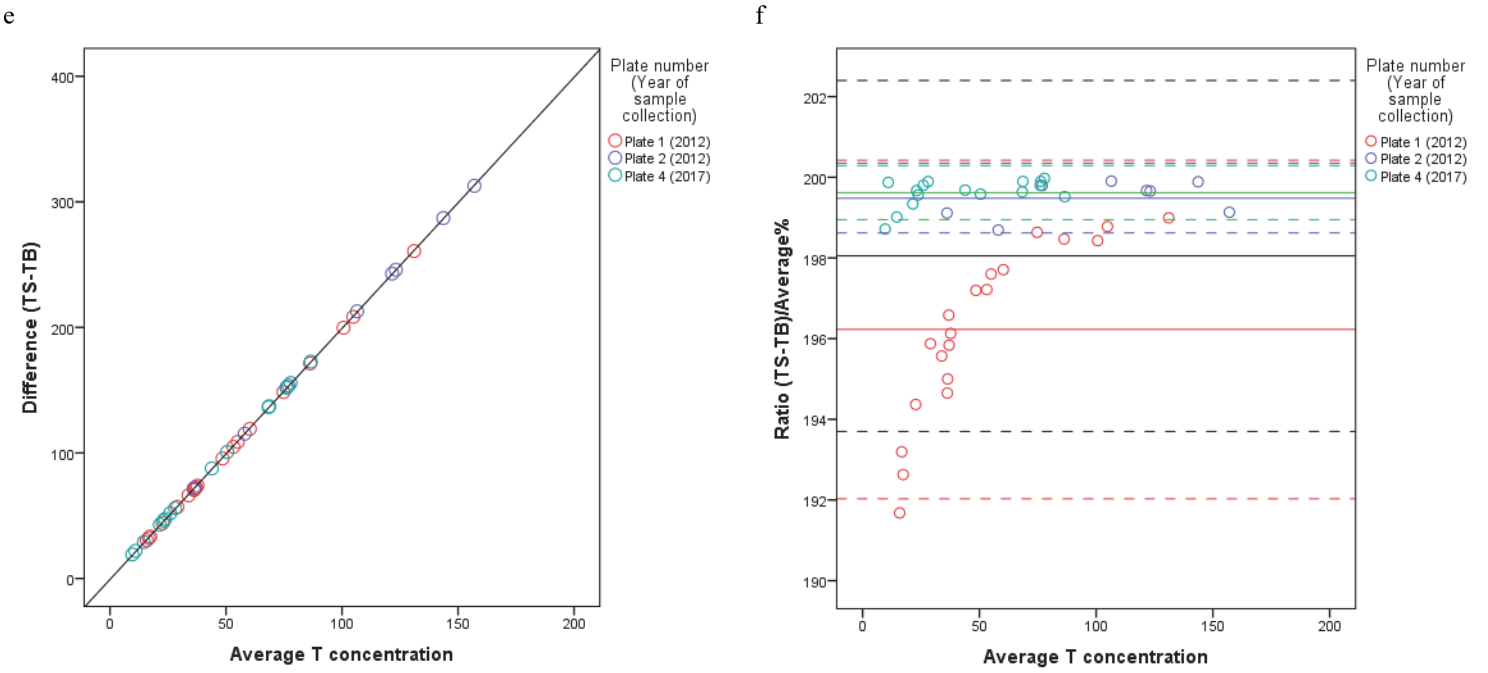


Fig. 2.14. Bland Altman plot with a) a relationship between absolute difference of testosterone (T) concentrations of two matrices and their mean concentration (different colors indicate different plates for plasma samples (Plate 1, Plate 2, Plate 4) with a linear regression line (r^2 = 1); b) a relationship of proportional ratio (%) of testosterone (T) concentrations of two matrices and their mean concentration (different colors indicate different plates for plasma samples (Plate 1, Plate 2; Plate 4), solid lines indicate mean bias and dashed lines indicate the 95% CI (mean +/- 1,96*SD) of mean bias).

2.3.6. Sex and nutritional stage effects in different matrices

Nutritional stage was the only significant effect in the model explaining variation in ES (AICc = 30,64 vs. $AICc_0$ = 56,23; $F_{3,23}$ = 14,44, p < 0,001): higher concentrations occurred during early and late suckling compared with the post-weaning fast (Fig. 2.15b).

Table 2.9. Generalized linear mixed model statistics for different steroid concentrations from saliva (ES) and plasma (EB).

ES				95% Confidence interval	
Fixed effects	$\beta \pm$ SE	t	p	Lower	Upper
Intercept	1.88 ± 0.14	13.09	< 0.001	1.59	2.18
Nutritional st. S1	1.24 ± 0.21	5.85	< 0.001	0.80	1.68
Nutritional st. S2	0.66 ± 0.21	3.12	< 0.01	0.22	1.10
Nutritional st. W1	0.12 ± 0.2	0.63	0.54	-0.28	0.531
Nutritional st. W2[a]	-	-	-	-	-

EB				95% Confidence interval	
Fixed effects	$\beta \pm$ SE	t	p	Lower	Upper
Intercept	92.53 ± 30.42	3.04	< 0.01	29.43	155.62
Sex (Female)	56.93 ± 27.08	2.10	0.047	0.77	113.10
Sex (Male)[a]	-	-	-	-	-
Nutritional st. S1	164.54 ± 38.60	4.26	< 0.001	84.49	244.60
Nutritional st. S2	102.57 ± 39.09	2.62	0.015	21.50	183.64
Nutritional st. W1	34.92 ± 35.92	0.97	0.341	-39.52	109.46
Nutritional st. W2[a]	-	-	-		

[a] *– redundant coefficients are set to zero*

The best model to describe variation in EB included two significant variables (AICc = 260,61 vs. $AICc_0$ = 314,83). EB was significantly different between nutritional stages ($F_{3,22}$ = 7,18, p < 0,01) and higher in females ($F_{1,22}$ = 4,42, p = 0,047). EB was highest during the early suckling period, remained lower and stable during late suckling – early post-weaning fast periods and dropped again during the late post-weaning fast (Fig. 2.15a). No significant interactions were observed.

Individual ID as a random intercept factor was removed, since it was not significant in any of the models (Table 2.9).

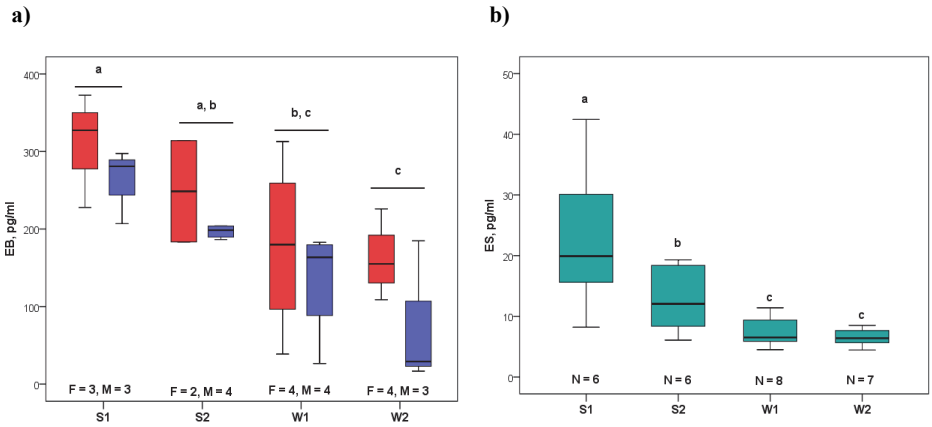


Fig. 2.15. Concentrations of EB (a) and ES (b) from grey seal pups during different nutritional stages (S1, S2, W1, W2). Red boxes (a) denote EB concentrations of female, blue – of male grey seal pups. Letters indicate significant ($p < 0.05$) differences between steroid concentrations during different nutritional stages. Middle line denotes median, outer box – interquartile range; whiskers – 95 % confidence intervals of steroid levels. Sample size (N) for each nutritional stage is given below each bar in the graphs. Different letters indicate significant differences ($p < 0,05$) between different nutritional stages in both graphs.

Two significant factors explained CS concentrations (AICc = 63,64 vs. $AICc_0$ = 89,36) – year of collection ($F_{1,52}$ = 14,48, $p < 0,01$) and nutritional period ($F_{1,52}$ = 18,42, $p < 0,001$). Higher concentrations were observed during the suckling period compared to fasting and in 2012 compared to 2017 (Fig. 2.16b). No interactions were observed. Although random effect was not significant, it substantially improved the model and was kept (Table 2.10).

The best model to describe variation in CB included two significant variables (AICc = 42,02 vs. $AICc_0$ = 101,04). CB was significantly different between the plates ($F_{2,51}$ = 29,05, $p < 0,001$) and nutritional period ($F_{1,51}$ = 8,03, $p < 0,01$). CB was highest when analyzed with Plate 1 (2014), however no difference was found between Plate 2 and Plate 4 (Fig. 2.16a). No significant interactions were observed. Individual ID as a random factor did not improve the model and was removed (Table 2.16).

Table 2.10. Generalized linear mixed model statistics for different steroid concentrations from saliva (CS) and plasma (CB).

CS				95% Confidence interval	
Fixed effects	$\beta \pm$ SE	t	p	Lower	Upper
Intercept	-2,31 ± 0,11	-20,69	< 0,001	-2,53	-2,08
Year of collection (2012)	0,53 ± 0,14	3,81	< 0,001	0,25	0,82
Year of collection (2017) [a]	-	-	-	-	-
Nutritional pr. Suckling	0,43 ± 0,1	4,34	< 0,001	0,23	0,63
Nutritional pr. Post-weaning fast [a]	-	-	-	-	-
Random intercept	*Estimate* ± SE	Z	p	Lower	Upper
Pup ID	0,05 ± 0,03	1,57	0,12	0,02	0,19

CB				95% Confidence interval	
Fixed effects	$\beta \pm$ SE	t	p	Lower	Upper
Intercept	3,78 ± 0,07	50,74	< 0,001	3,63	3,93
Plate 1 (2014)	0,65 ± 0,1	6,59	< 0,001	0,45	0,85
Plate 2 (2015)	-0,14 ± 0,12	-1,14	0,26	-0,39	0,11
Plate 4 (2018) [a]	-	-	-	-	-
Nutritional pr. Suckling	0,26 ± 0,09	2,8	< 0,01	0,8	0,45
Nutritional pr. Post-weaning fast [a]	-	-	-	-	-

[a] – *redundant coefficients are set to zero*

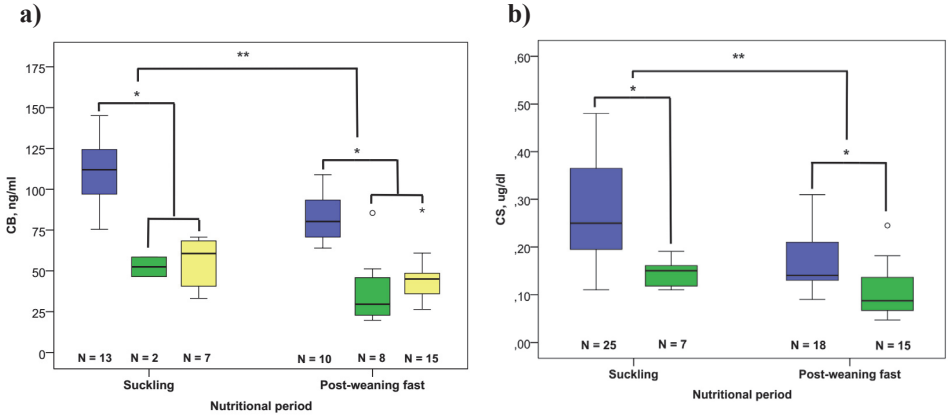


Fig. 2.16. Concentrations of CB (a) and CS (b) from grey seal pups during different nutritional period (Suckling and Post-weaning fast). Different colors (a) of CB boxes denote different ELISA plates (blue – Plate 1 (2014), green – Plate 2 (2015), yellow – Plate 4 (2008)), and different colors (b) of CS boxed denote different concentration during different sample collection year (blue – 2012, green – 2017). * indicate significant ($p < 0,05$) differences of steroid concentrations between plates/ years of sample collection; ** - indicate significant differences ($p < 0,05$) of steroid concentrations between nutritional periods. Middle line denotes median, outer box – interquartile range; whiskers – 95 % confidence intervals, circles – outliers, stars - extremes of steroid levels. Sample size (N) for each nutritional period is given below each bar in the graphs.

2.3.7. Comparison between steroid concentrations obtained with ELISA and alternative methods (RIA, UPC_2-MS/MS)

Median EB concentration 9,06 pg/ml (IQR = 7,28, min = 0,64, max = 40,17) was about ten times smaller than median concentration of EB_RIA – 128,16 pg/ml (IQR = 64,91, min = 62,34, max = 265,68) (mean difference EB_RIA vs. EB – 116,38 ± 44,94 pg/ml, t = 14,42, df = 30, p < 0,001). ICC coefficient was positive, but not significant for both single ICC = 0,26 (95% CI = -0,09 – 0,56) and average ICC = 0,42 (95% CI = -0,21 – 0,72) measures ($F_{30,30}$ = 1,72, p = 0,07). Cronbach's alpha is 0,42. Good correlation (N = 31) was observed between concentrations from neat (EB_RIA) and processed (EB) samples (Fig. 2.17).

Median CB concentration 46,61 ng/ml (IQR = 26,27, min = 22,21, max = 87,2) was about as twice larger than median concentration of COR – 21,58 ng/ml (IQR = 14,43, min = 5,53, max = 54,94) (mean difference CB vs. COR – 27,16 ± 10,2 ng/ml, t = 14,82, df = 30, p < 0,001). ICC coefficient was positive significant for both single ICC = 0,73 (95% CI = 0,51 – 0,86) and average ICC = 0,84 (95% CI = 0,68 – 0,93) measures ($F_{30,30}$ = 6,4, p < 0,001). Cronbach's alpha is 0,84. Very good correlation (N = 31) was observed between concentrations from neat (CB_RIA) and processed (CB) samples (Fig. 2.18).

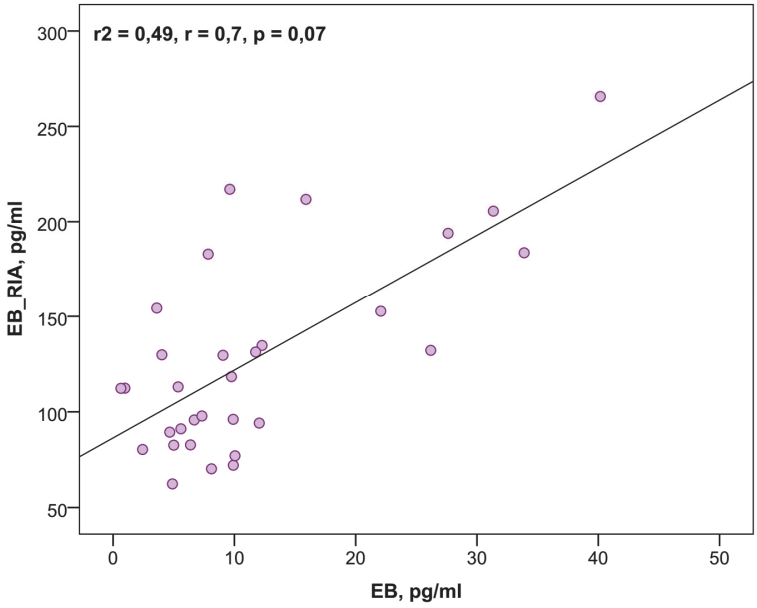


Fig. 2.17. Linear relationship between cortisol concentrations obtained by ELISA (EB) and RIA (EB_RIA) methods. Regression (r^2) and correlation coefficients (r), and significance values (p) are shown on the graph. Black line indicates linear trend.

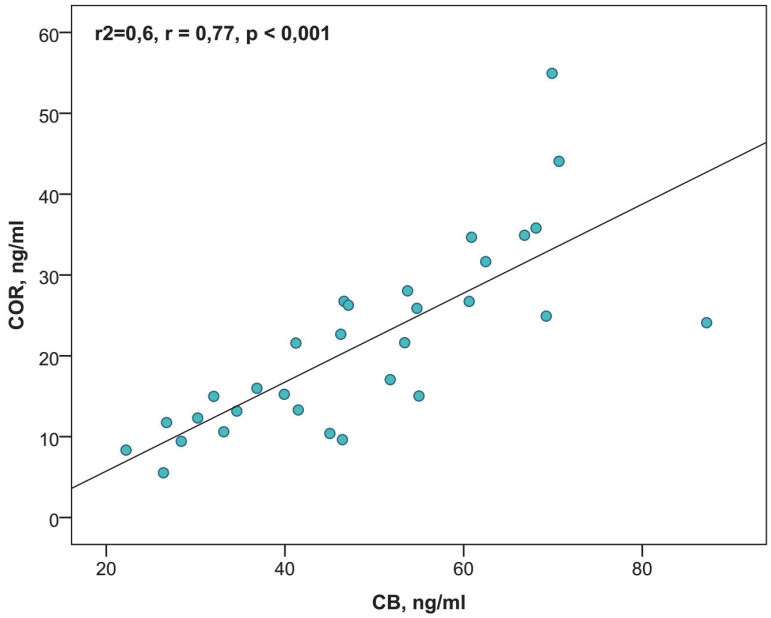


Fig. 2.18. Linear relationship between cortisol concentrations obtained by ELISA (CB) and UPC_2-MS/MS (COR) methods. Regression (r^2) and correlation coefficients (r), and significance values (p) are shown on the graph. Black line indicates linear trend.

2.3.8. Concentrations of steroids measured by UPC_2-MS/MS and RIA

Concentrations between 15 steroids determined by UPC_2-MS/MS varied a lot (Fig. 2.19). The largest concentrations (around 10-20 ng/ml) were of glucocorticoids and mineralocorticoids – cortisol (COR), cortisone (CORNE), corticosterone (COS). Their precursors – 11-deoxycortisol (11-deoxyCOR) and deoxycorticosterone (DOC) were in much lower levels. Androstenediol (A5) was the most abundant androgen (~10 ng/ml), however other androgens had lower concentrations, most of them < LOQ. Pregnenolone (PREG) was the most abundant progestogen and varied around 5 ng/ml. Estrone (E1) was the only estrogen detected by the method and its concentration varied around 2 ng/ml. TB_RIA had very low concentrations, which resembled those of TB, analyzed by ELISA (Table 2.11).

Significant concentration differences ($p < 0,05$) between different nutritional stages (S1, S2, W1, W2) were detected in glucocorticoids COR, CORNE, DOC and testosterone (TS) (Table 2.11). Significant differences

between pups of different sex were seen in concentrations of androstenedione (AN) and EB_RIA. Indeed, AN was observed above LOQ in female pups (n = 11) and only one sample from male pups (N = 20) had this concentration above LOQ.

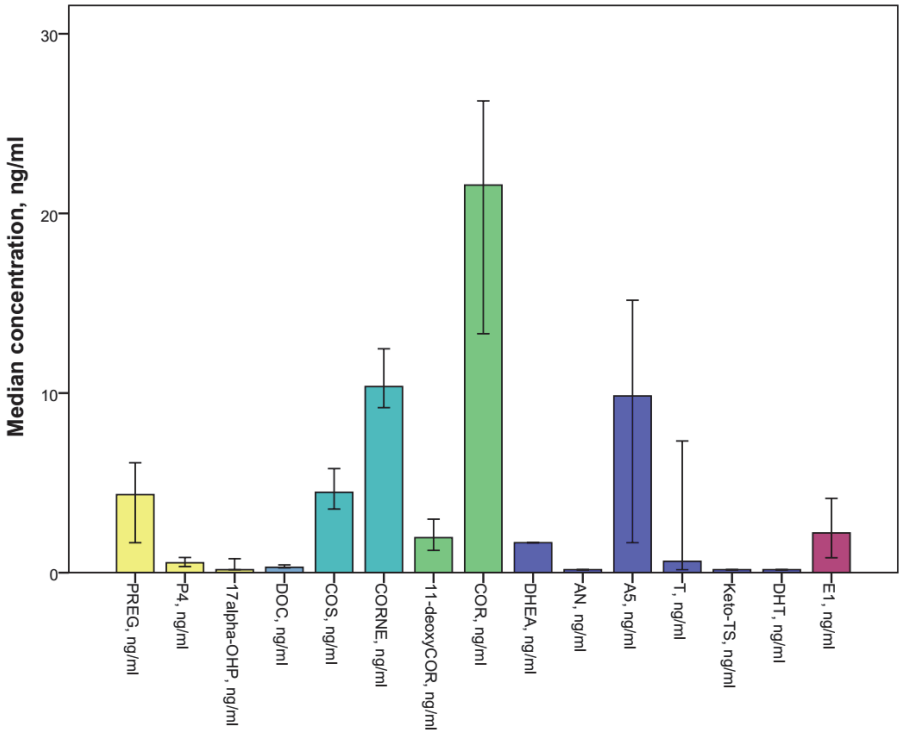


Fig. 2.19. Median concentrations (with 95% Confidence intervals) of different steroids analyzed with UPC_2-MS/MS from grey seal pup plasma during suckling and post-weaning fast (N = 31). Colors represent different classes of steroids: yellow – progestogens, light blue – transitional steroid between glucocorticoids and mineralocorticoids, green – glucocorticoids, dark blue – androgens, purple – estrogens. Abbreviations are explained in Table 2.11.

Table 2.11. Mean concentrations (± SD) of different steroids analyzed by UPC_2-MS/MS and RIA during different nutritional stages (S1, S2, W1, W2) and for different sex individuals (F - females, M - males). Significance values ($p < 0,05$), calculated with nonparametric Kruskal-Wallis (KW) and Mann-Whitney (MW) tests, are bolded and marked with asterisk.

	Nutritional stages					Sex		
Steroid (Abbreviation)	**S1**	**S2**	**W1**	**W2**	**KW test**	**F**	**M**	**MW test**
UPC_2-MS/MS (ng/ml)								
n	9	7	9	6		11	20	
Cortisone (CORNE)	**14,95 ± 5,76**	**12,49 ± 1,85**	**9,85 ± 3,63**	**8,74 ± 1,82**	**p = 0,02***	10,14 ± 3,28	12,57 ± 4,82	p = 0,12
Corticosterone (COS)	5,81 ± 3,43	4,68 ± 4,27	4,29 ± 1,49	5,42 ± 2,3	p = 0,6	4,48 ± 2,59	5,34 ± 3,14	p = 0,45
11-deoxycortisol (11-deoxyCOR)	2,92 ± 2,11	1,56 ± 1,64	2,66 ± 1,22	1,08 ± 1,31	p = 0,096	2,69 ± 2,02	1,9 ± 1,52	p = 0,3
11-deoxycorticosterone (DOC)	**0,48 ± 0,18**	**0,43 ± 0,2**	**0,27 ± 0,13**	**0,26 ± 0,1**	**p = 0,045***	0,33 ± 0,18	0,38 ± 0,19	p = 0,5
Pregnenolone (PREG)	6,02 ± 4,42	10,77 ± 12,51	2,67 ± 1,6	5,46 ± 2,05	p = 0,16	4,72 ± 3,34	6,72 ± 8,16	p = 0,92
Progesterone (P4)	1,14 ± 0,99	0,83 ± 0,51	0,47 ± 0,43	0,4 ± 0,23	p = 0,086	0,89 ± 0,97	0,65 ± 0,47	p = 0,7
Androstenedione (AN)	0,27 ± 0,17	1,37 ± 3,18	0,36 ± 0,43	0,25 ± 0,13	p = 0,95	**1,21 ± 2,47**	**0,18 ± 0,04**	**p < 0,01***
Androstenediol (A5)	11,31 ± 12,85	11,15 ± 7,77	7,45 ± 9,34	13,5 ± 11,45	p = 0,69	10,95 ± 11,26	10,37 ± 10,02	p = 0,98
Testosterone (TS)	**8,11 ± 6,6**	**0,17 (LOQ/2)**	**6,61 ± 9,63**	**5,48 ± 5,46**	**p < 0,01***	2,5 ± 4,44	5,91 ± 8,03	p = 0,92
11-ketotestosterone (KetoTS)	0,19 ± 0,06	0,27 ± 0,18	0,17 (LOQ/2)	0,35 ± 0,3	p = 0,23	0,2 ± 0,11	0,25 ± 0,19	p = 0,64
5α-dihydrotestosterone (DHT)	0,36 ± 0,24	0,17 (LOQ/2)	0,3 ± 0,29	0,36 ± 0,24	p = 0,22	0,29 ± 0,22	0,3 ± 0,24	p = 0,95
Dihydroepiandrosterone (DHEA)	6,64 ± 13,32	5,7 ± 7,44	2,41 ± 1,48	2,27 ± 1,47	p = 0,67	4,71 ± 5,93	4,16 ± 9,02	p = 0,24
17α-hydroxyprogesterone (17α-OHP)	0,68 ± 0,58	0,97 ± 1,1	0,51 ± 0,7	0,27 ± 0,16	p = 0,37	0,88 ± 0,94	0,47 ± 0,55	p = 0,16
Estrone (E1)	3,4 ± 2,56	3,29 ± 2,34	3,26 ± 3,34	1,82 ± 2,43	p = 0,42	1,99 ± 2,33	3,6 ± 2,74	p = 0,066
RIA								
17β - Estradiol (EB_RIA), pg/ml	158,84 ± 54,45	126,02 ± 65,18	119 ± 41,32	98,38 ± 19,2	p = 0,15	**156,89 ± 50,96**	**112,35 ± 45,45**	**p < 0,01***
Testosterone (TB_RIA), ng/ml	0,02 ± 0,02	< LOD	0,01 ± 0,002	< LOD	p = 0,21	0,05 ± 0,07	0,03 ± 0,02	p = 1

2.3.9. Links between the behavior and steroid hormones during suckling and post-weaning fast periods

Resting (R) behavior was the most dominant in pup's behavioral budget during suckling, followed by resting comfort moves (R-CM) and suckling (Skl). Proportions of behaviors differed significantly during the suckling period (Friedman's $\chi 2 = 42{,}51$, $df = 10$, $p < 0{,}001$). After Dunn's correction significant differences were observed between proportions of R, R-CM, Skl in comparison with Ag, Voc and NP (Fig. 2.20). Overall social behavior made about 20 % of total behavioral repertoire. No sex difference was seen among behavior between different sex individuals during suckling period (MW, $p > 0{,}05$).

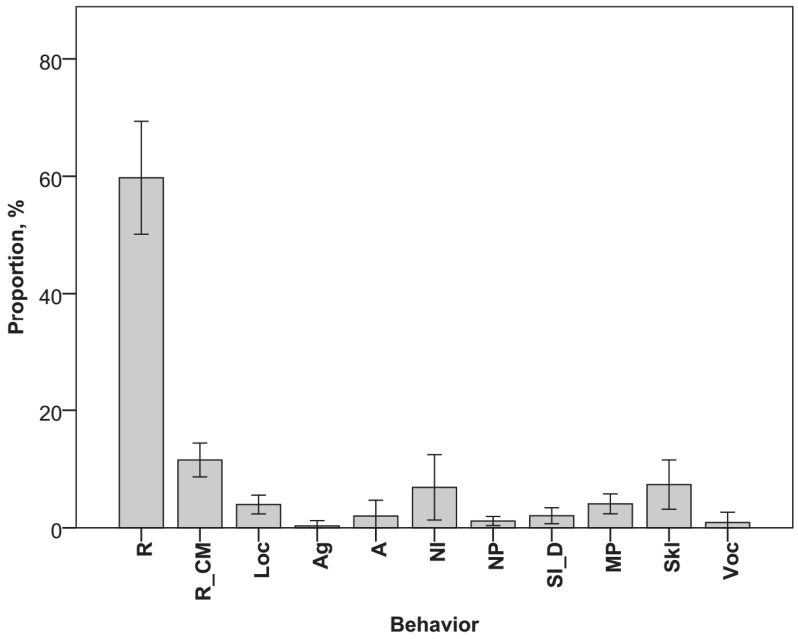


Fig. 2.20. Individual (n = 5) proportion of scans (%) of particular behavior during the suckling period. Medians with 95 % confidence intervals are provided in the graph (Friedman's $\chi 2 = 42{,}51$, $df = 10$, $p < 0{,}001$). Abbreviations are explained in Table 2.5.

Significant correlations between behavior and steroids during the suckling period (n = 5) were identified using Spearman rank correlation (Fig. 2.21). Strong positive correlation was found between R and 11-deoxycortisol (11-deoxyCOR, $r = 0{,}9$, $p = 0{,}037$), while opposite negative correlation – with progesterone (P4, $r = -0{,}9$, $p = 0{,}037$). A negative significant correlation was found between pregnenolone (PREG) and alert (A, $r = -0{,}9$, $p = 0{,}037$), while

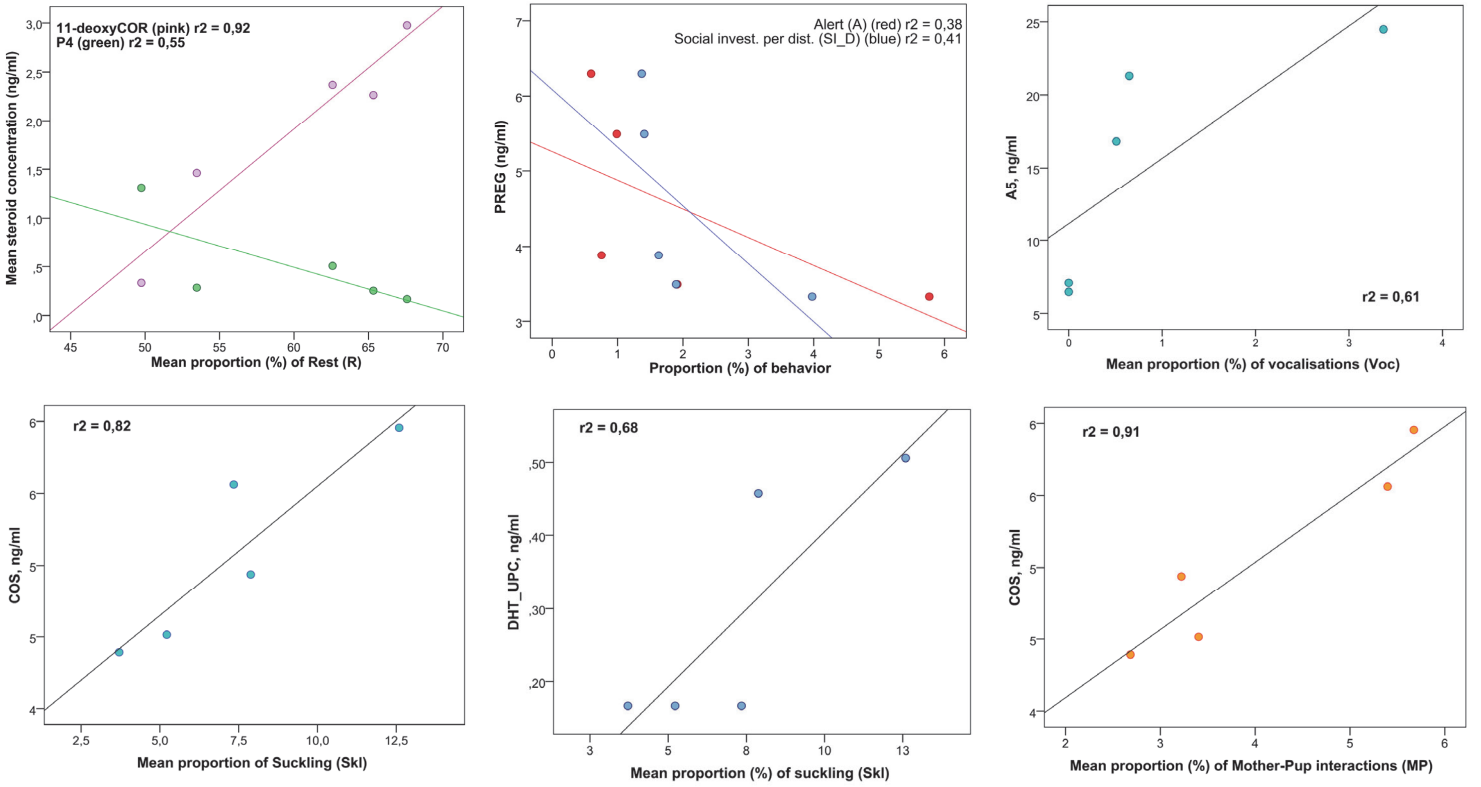


Fig. 2.21. Correlation parameters (r – Spearman rank correlation coefficient, p – statistical probability value, n – sample size) between different mean proportion (%) of behavioral elements and mean concentrations of different steroids for same individuals during suckling period (n = 5). Coefficient of regression (r^2) is indicated on the graph.

cortisone (CORNE, r = 0,9, 0,37). Corticosterone (COS) correlated positivelu with both – suckling (Skl) and mother-pup interaction (MP) (r = 0,9, p = 0,037). Alert had a positive (r = 0,89, p = 0,04) and suckling had a negative correlation (r = -0,89, p = 0,04) with dehydrotestosterone analysed with UPC_2-MS/MS (DHT). Androstenediol (A5) had a strong positive correlation (r = 0,95, p = 0,23) with proportions of vocalization (Voc). No significant correlations (p < 0,05) were found between body mass (kg) and average age (days) during sampling procedure during suckling period neither with proportion of behavior, nor concentrations of different steroid hormones.

Alert (A), non-social investigation (NI) and rest (R) were most often observed behaviors (n = 5) during post-weaning fast (Fig. 2.22). Significant differences were found between proportions of different behaviors (Friedman's $\chi 2 = 36,59$, df = 8, p < 0,001). After Dunn's correction significant differences were found between NI and SI_C, Voc and SI_D. In addition, proportions were different between SI_C and R, and between SI_D and R. Social behavior made around 10 % of the total behavior repertoire. No sex difference was seen among behavior between different sex individuals during the post-weaning fast period (MW, p > 0,05).

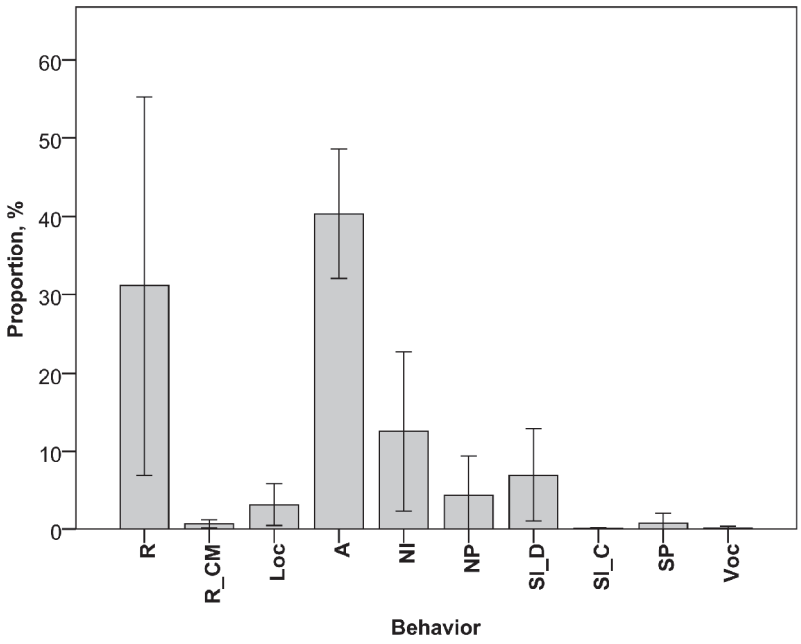


Fig. 2.22. Individual (n = 5) proportion of time duration (%) of particular behavior during the post-weaning fast period. Medians with 95 % confidence intervals are provided in the graph. Abbreviations are explained in Table 2.5.

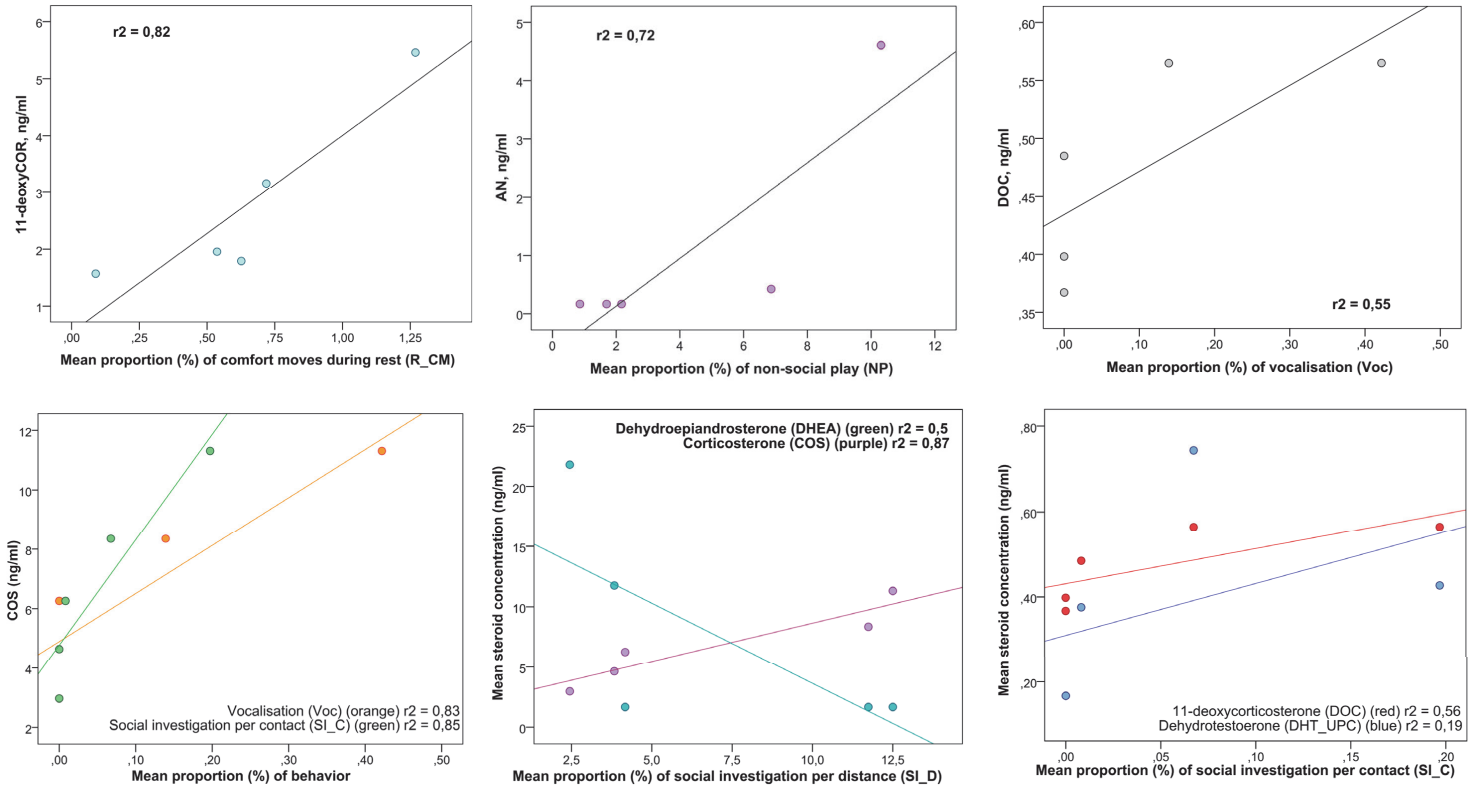


Fig. 2.23. Correlation parameters (r – Spearman rank correlation coefficient, p – statistical probability value, n – sample size) between different mean proportion (%) of behavioral elements and mean concentrations of different steroids for same individuals during suckling (n = 5) of an individual seal.

Same Spearman rank correlation was used to identify significant correlations between behavior and steroids during the post-weaning fast period (n = 5) (Fig. 2.23). Strong positive correlation was found between R_CM and 11-deoxycortisol (11-deoxyCOR, r = 0,9, p = 0,037). Both 11-deoxycorticosterone (DOC) and corticosterone (COS) correlated identically positively with social investigation per contact (SI_C) (r = 0,98, p < 0,01), social contact per distance (SI_D) (r = 1, p < 0,001), and vocalization (Voc) (r = 0,89, p = 0,04). In addition, proportion of SI_C had a positive (r = 0,9, p = 0,04) with dehydrotestosterone analyzed with UPC_2-MS/MS (DHT), while SI_D had a negative correlation with dihydroepiandrosterone (DHEA) (r = -0,89, p = 0,04). Androstenedione (AN) had a strong positive correlation (r = 0,89, p = 0,04) with proportions of mean proportion of non-social play (NP). This time significant positive correlations were found between mean pup body mass (kg) and Voc (r = 0,89, p = 0,04), and negative with R_CM (r = -0,9, p = 0,04) and 17α-hydroxyprogesterone (17α-OHP) (r = 0,9, p = 0,037). No correlation was found between mean age (days) during sampling procedure during post-weaning period neither with proportion of behavior, nor concentrations of different steroid hormones.

2.4. DISCUSSION

2.4.1. Steroids in saliva and plasma

Measurement of steroids in wild pinnipeds can facilitate assessment of breeding, nutritional and stress status (Bennett et al., 2013; Champagne et al., 2016; Kershaw and Hall, 2016; Meise et al., 2016), and is useful in understanding behavioral responses (Klein et al., 1997; Lidgard et al., 2008). Saliva has never been used to assess steroid levels in pinniped species in the wild. Here an attempt to measure three steroids in saliva from wild grey seal pups using commercial ELISA kits was made. Saliva was easier to work with after collection, and saliva concentrations of cortisol and estradiol correlated well with values in plasma. However, differences in correlation of estradiol levels between the plates could be explained by very low concentrations of plasma estradiol in some years which reduces the sensitivity of correlation. Testosterone plasma assays performed less well, which prevented testing of the relationship between plasma and saliva levels. Importantly, the success of saliva sample collection was low, even from anesthetized animals, compared to blood collection. Lengthier sample collection time and unpredictability in obtaining samples limited the utility of saliva in assessing steroid levels in wild seals.

2.4.1.1. Ease and success of saliva sample collection and sample preparation

The ease and success of sample collection and sample preparation of two different matrices for steroid analysis was compared (Table 2.12). Saliva sample preparation was faster by up to one day and simpler – required less equipment, chemicals and elaborate steps of extraction, than preparation of plasma samples. However, while saliva may therefore appear to be an attractive option for monitoring E and C in grey seals, there are a number of caveats. Firstly, saliva samples take substantially longer (up to ten times) to collect than plasma samples in wild grey seal pups, even when, as here, the animals are anaesthetized. Since this type of anesthesia still requires venipuncture the involvement of a trained professional is required to administer the anesthetic agent and obtain the sample, venipuncture is not avoided and there is risk of extended apnea from the anesthesia. In addition, the longer contact time may incur greater stress and therefore negates the use of saliva as an alternative matrix. In unanesthetized animals handling time and

Table 2.12. Sampling and processing qualities of saliva and plasma collection for further steroid analysis using ELISA. Letter P indicates Positive aspects and N – negative aspects of collection of different matrix samples.

Quality	Saliva	Plasma
Ease	P: No venipuncture necessary Little risk of infection N: Anesthesia or sedation required if collected in the wild Longer duration for sample collection (up to 10 min.) Likely stressful for an animal and challenging for handler when animal is not anesthetized	P: Quick N: Venipuncture necessary Requires an experienced technician to reduce the time of sample collection and possible handling stress and damage Possible infection risk
Success	N: ~ 60 % sampling success (minimum volume collected) 40 % likelihood of not obtaining the sample due to blood contamination and low saliva productivity Limited volume (sufficient volume for 3 assays 84 %), therefore depends on the number of assays to be performed and minimum volume may not be obtained High probability of environment contamination	P: 100 % success High volume from one sampling effort Low probability of environment contamination
Processing	P: Short processing	N: Longer processing if steroid extraction from the sample is necessary Use of chemicals and equipment for extraction is more cost demanding

stress, and the risk of gum damage if the animal struggles leading to blood contamination, will be substantially greater. In addition, it was often difficult to obtain enough sample for all three steroids to be analyzed: it was only possible to obtain saliva in 60 % of sampling events, compared to 100 % for blood collection and 84 % reached a recommended volume of 0,5 ml or more, and only 3 % of samples reached a volume of 1,5 ml – a recommended 0,5 ml volume for every assay (Supplementary Fig. S1). Theodorou and Atkinson (1998) reported that anesthetized animal produced significantly lower volume of saliva, not sufficient for steroid analysis, which might be the case here. Saliva may therefore be more appropriate for use in species that will tolerate mouth swabbing, in captive individuals that have been trained to accept saliva

sampling, or where anesthesia is deemed appropriate and safe for the species and age group, and if sufficient sample is guaranteed to be obtained for the analysis. These conditions are less likely to be met in a wild context. Our study shows that there is no guarantee that sufficient and uncontaminated saliva sample will be collected to complete an intended analysis even when animals are anesthetized and when adequate time is given for saliva collection.

2.4.1.2. Utility of commercial ELISA kits for steroid analysis from grey seal saliva and plasma

This study demonstrates that estrogen and cortisol can be measured with acceptable accuracy in both plasma and saliva of grey seal pups: assay performance was from acceptable (~50-70 %) to excellent (~100 %) and there was a positive correlation between plasma and salivary levels. However, if cortisol had a strong and constant positive correlation between two matrices that could be observed using different plates and between the years, a greater variation between the plates and years was seen when comparing levels of estradiol between two matrices. It seems that lack of correlation can be seen when levels of plasma steroids are very low, indicating that when concentrations falls around the limit of quantification, the changes in saliva might not be reflected in plasma. In addition, saliva estradiol concentrations have a negative relationship with saliva volume, which could again affect the relationship with plasma levels. However, even though EB seems to have an excellent inter-assay CV, the average concentration of samples from the second plate was about 40-50 % lower. For CB, lack of specificity due to high cross reactivity with progesterone and its metabolites could be one of the reasons for such results. While T assay dilution performance and recovery rates for saliva and blood was from acceptable to excellent, the repeatability of TB concentrations was very poor, with average inter-assay CV reaching 86,24 %. It is hard to identify what could have caused these differences between plates analyzed at different times. Steroids could have degraded, but are considered to remain stable at -20^0C for up to 40 years (Henderson et al., 1988; Stroud et al., 2009; Zhang et al., 2007). There could also be changes in antibody performance between plate batches, which was hard to determine with limited sample to work with. A pooled sample should be made up to cover multiple plates to account for differences between batches. Significant variability between concentrations of plasma testosterone, extracted with tert-butyl methyl ether and evaluated using commercial ELISA kits in different

years was also found in a study with wild red deer (*Cervus elaphus*) calves (Pavitt et al., 2014). They suggest that fluctuations in laboratory environment could have a significant impact on assay performance. Some differences could have been introduced by small, unintentional differences in extraction. Low sensitivity of competitive ELISA has been discussed in some previous studies (Schrijver and Kramps, 1998), as well as limited reproducibility (Kinn Rød et al., 2017). Limited assay sensitivity combined with the low concentrations in pups could have produced high variation between the plates (Holder et al., 2010; Stanczyk et al., 2003). Commercial ELISA kits may thus be suitable for estimating estradiol and cortisol in grey seal pup plasma, but efforts need to be made to minimize between plate variation and ensure plate design across study is well planned prior to the assay to minimize potential inter-plate confounds, such as ensuring serial samples from the same individuals are measured in the same plate. However, small differences, such as individual variation in young pups might be too small to detect, especially when intra and inter assay variability are high.

Another problem here was a large number of samples < LOD for plasma estradiol and testosterone, especially from later sampling years (2016, 2017). Cortisol concentrations were lower in the later sampling year, but because concentrations are high in general, ranging around 50 ng/ml after extraction, the extraction procedure does not reduce them to values below LOD. While estradiol is measured in tens of picograms per milliliter, which means that extraction procedure can significantly affect concentrations, especially if they are lower. Thus effort to measure plasma estradiol without extraction or reducing the volume of the assay buffer would be recommended. There are several ways to deal with samples that are below LOQ, from discarding to including same standardized concentrations LOQ/2. However, according to Keizer et al. (2015) experiment, inclusion of such samples in their original concentration is much more beneficial than excluding or transforming these concentrations, because they provide better overall model estimates.

No relationship was found between TB and TS. This contrasts with the strong correlation in these two matrices in adult Hawaiian monk seals (*Neomonachus schauinslandi*) (Theodorou & Atkinson 1998), but conforms to the lack of a positive correlation in several other animal species (Cadore et al., 2008; Gröschl, 2008). While it is possible that the salivary samples were contaminated during sample collection given the possibility for unnoticed grass or dirt to get in the animals' mouths at the time of collection, a more

likely explanation is that there may be differences in interfering substances between saliva and plasma, such as transport proteins (Wong et al., 1992). The concentrations of steroid sulfates in human newborns are about 10 times higher than in the maternal blood and this difference is seen long into infancy (Shackleton et al., 1979; Wong et al., 1992). Steroid sulfates can interact with plasma ELISA and provided inaccurate results in young individuals (Wong et al., 1992) that do not correlate with free steroid concentrations in saliva. Although the extraction procedure should remove plasma steroid metabolites (Stanczyk et al., 2003), some may be more resistant and could interfere in the ELISA if they are not efficiently removed during processing. Moreover, steroids are soluble in organic solvents as are lipids, therefore in highly lipemic blood of a pup, some steroids could have dissolved in the lipid layer that is extracted which does not allow steroid to bind to a binding site or can interfere with ELISA itself (Ferraz et al., 2004; Nikolac, 2014). Lipemia could be the reason for variability of steroid concentrations between the plates and an alternative steroid extraction method that would remove the majority of lipids without removing steroids (Ferraz et al., 2004), could be used. Enzyme immunoassays can often provide inaccurate results when low concentrations of testosterone are in the matrix, for example in postmenopausal women or children (Stanczyk et al., 2003; Taieb et al., 2003; Wood, 2009). In addition, an evidence that saliva T concentrations were negatively related to sample volume was found, even though T should not be related to salivary flow rate (Wood, 2009). Therefore, it is not possible to distinguish whether the TS data in this study is an artefact of small sample amount or reflected a relationship between T and saliva flow rate. Lack of correlation between T concentrations in the two matrices might indicate that pups differ from adult seals in circulating metabolites and interfering substances, and/ or that the kit specificity and sensitivity was not adequate for this application (Durdiaková et al., 2013; Granger et al., 2004).

2.4.1.3. Steroid concentration sensitivity between different matrices

Saliva provided similar detail on estradiol and cortisol differences between nutritional states compared with plasma. However, no sex differences were observed in saliva, whereas females consistently had higher estradiol than males in the matched plasma samples. These early sex differences have not been previously reported. The lack of detectable sex differences in saliva estradiol may limit its utility for measuring sex steroids in young animals.

Despite concentration differences of cortisol between the plates, similar detail was provided from both – saliva and plasma matrices, showing that any matrix is suitable for identification of cortisol in grey seal pups during suckling and post-weaning fast.

2.4.1.4. Steroid comparability between different methods

Plasma cortisol was highly comparable between two different methods (ELISA and UPC_2-MS/MS). However, estradiol concentrations between two different methods – ELISA and RIA – even though positive, the relationship was not significant. One of the reasons for lack of comparability of estradiol concentrations might be very low values, which only shows that low values lack the sensitivity power. It was impossible to compare testosterone concentrations between ELISA and RIA due to very low concentrations from 2017 samples. Even though a more sensitive analysis method would be recommended for testosterone in grey seal pups, investigation of neat concentrations and extraction efficiency for estradiol using same kits could be the next step for future research.

The performance of UPC_2-MS/MS allowed investigation of multiple steroids in great detail and with confidence at the same time, however it requires substantial preprocessing and analysis time, requires experienced staff and substantial costs. Additionally, it is not as sensitive as RIA or ELISA when measuring concentrations such as estrogens or aldosterone that are reported to be in pg/ml rather than ng/ml in several pinniped species outside the breeding season (Ferreira et al., 2005; Lydersen and Kovacs, 2005; Reijnders, 1990). However, it was possible to detect estrone, which should be the most dominant estrogen in immature individuals (Ruiz-Cortes, 2012) and was also detected in adult captive Hawaiian monk seal female at ranges from almost zero concentrations outside the breeding season to 10 ng/ml during the peak ovulation (Pietraszek and Atkinson, 1994). According to the latter study, estrone is the most common estrogen at least in Hawaiian monk seals.

2.4.2. Steroid concentrations in comparison to other studies

2.4.2.1. Saliva steroids

Saliva steroid concentrations measured here were comparable with those reported previously in other marine mammal groups (Amaral et al., 2015; Theodorou and Atkinson, 1998). The testosterone concentrations of captive

Amazonian manatee (*Trichechus inunguis*) males were around 31 pg/ml (Amaral et al., 2015) and those of Hawaiian monk seals were around 30-190 pg/ml (Theodorou and Atkinson, 1998). Even saliva steroid concentrations (0,3-1,2 ng/ml) of adult male Steller sea lion (*Eumetopias jubatus*) were comparable with those reported in this study. Estradiol concentrations from captive Amazonian manatee females obtained via enzyme immunoassay (EIA) varied around 2-10 pg/ml (Amaral et al., 2015), which is lower than concentrations found in our study. Most reported saliva cortisol values are those of captive bottlenose dolphins (*Tursiops truncatus*), in which concentrations are rather low, varying from 0,0042 ng/ml to 0,55 ng/ml (Ugaz et al., 2013). Cortisol concentrations in a recent study of wild female and pup Antarctic fur seal (*Arctocephalus gazella)* obtained using ELISA were comparable to concentrations presented in this study and varied around 5-10 ng/ml (Nagel et al., 2022). The majority of previously reported testosterone and estrogen concentrations in saliva were measured by radioimmunoassay (RIA), however the range of salivary T and E values reported for different ages and sexes in marine mammals is broadly similar irrespective of analytical technique.

2.4.2.2. Plasma steroids

Plasma steroid concentrations are also comparable with previous studies in pinniped species, however the range of steroid concentrations in blood vary more between studies than concentrations reported in saliva. As with saliva, blood steroids are mostly analyzed using RIA (Table S1). Steroid concentrations obtained using UPC2-MS/MS are very similar to those of Bottlenose dolphins (*Tursiops truncatus*) obtained with liquid chromatography tandem mass spectrometry (Galligan et al., 2018a).

TB concentrations, measured using ELISA, are similar to those found in young and adult harbor seals (*Phoca vitulina*) (Lydersen and Kovacs, 2005) and southern elephant seals (Ferreira et al., 2005), as well as in non-breeding Weddell seals (*Leptonychotes weddellii*) (Bartsh et al., 1992) and bottlenose dolphins (Galligan et al., 2018a).

EB was higher than concentrations reported in harbor seal young and adults by Lydersen & Kovacs (2005), northern fur seals (*Callorhinus ursinus*) (Dierauf and Gulland, 2001) and southern elephant seals (*Mirounga leonina*) (Ferreira et al., 2005), but was similar to concentrations in harbor seal reported by Reijnders (1990). Estrone concentrations in grey seal pups are higher than those reported in Hawaiian monk seals outside the breeding season, but lower

than those during the peak ovulation of Hawaiian monk seals (Pietraszek and Atkinson, 1994) and spotted seals (Zhang et al., 2014). Progesterone in this study was around 1 ng/ml and resembled those of Hawaiian monk seals outside the breeding season, but there up to 60 times lower than those of harbor seal females at the end of parturition (Gardiner et al., 1999). Similarly 11α-OHP was around 1 ng/ml and resembled concentrations of female grey (Boyd, 1984) and spotted (Zhang et al., 2014) seals at parturition, but were up to ten times lower comparing to same females at final months of pregnancy.

Circulating cortisol concentrations found here were also similar to previous studies in suckling and weaned grey seal pups (Bennett et al. 2012) and to baseline cortisol concentration in adult males (Lidgard et al. 2008). In both of these studies, blood samples were obtained within 2-4 minutes of initial approach of the animal and varied from 21-37 ng/ml in weaned pups to 170 ng/ml in adult males. Similar concentrations of circulating cortisol to those in this study were found in Southern elephant juvenile and adult seals (Ferreira et al., 2005) and adult male Weddell seals (Bartsh et al., 1992). Several times higher cortisol concentrations were found in adult males Weddell seals in another study, which differed from previous that plasma samples instead of serum was collected (Harcourt et al., 2010).

2.4.3. Steroid concentration differences between sex and nutritional stages

This study shows that higher concentrations of estradiol in saliva and plasma during suckling than fasting reported in 2012 samples and higher concentrations of 11-deoxycorticosterone and cortisone from 2017 plasma samples may result from a reduction in metabolic processes after weaning when animals need to conserve energy (Reilly, 1991). It is also likely that steroid hormones, especially sex steroids, are not metabolized rapidly after birth and still resemble concentrations transferred from mother *in utero* (Ruiz-Cortes, 2012) or are transferred to pups through mother's milk (Pundir et al., 2020). Pregnenolone and progesterone concentrations drop dramatically fivefold in newborn foals over 24 hours after birth, and have a 4 hour half-life, while 11-deoxycorticosterone and dehydroepiandrosterone have a longer half-life of around 20 hours and remain elevated for several days (Aleman et al., 2019). A similar trend was observed with testosterone in newborn wild red deer (*Cervus elaphus*) calves, in which testosterone dropped significantly within 24 hours after birth (Pavitt et al., 2014). It is also possible that steroids,

as well as gonadotropin releasing hormones, which might contribute to elevated steroid concentrations, are transferred to pups through mother's milk (Corso et al., 2020; Pundir et al., 2020). Steroid levels may track changes seen in the mother, which have high concentrations of estradiol at birth followed by a drop postpartum and increasing levels during the second half of lactation, possibly due to estrus (Mellish and Iverson, 2005; Schweigert and Schams, 1993).

Although cortisol is a steroid very sensitive to capture stress and thus interpretation of the changes of this steroid during different nutritional stages should be looked at with caution, the general pattern of saliva cortisol reflected that reported in the wild Antarctic fur seal pups (*Arctocephalus gazella*) by Nagel et al. (2022). In the latter study both mothers and pups had considerably higher salivary cortisol concentrations early in the suckling period. Lower cortisol concentrations after a suckling period were also observed in other pinniped species and is related to a reduced metabolic processes and weight loss during the post-weaning fast (Bennett et al., 2013; Kelso et al., 2012). In this study we show the change not only in cortisol concentrations throughout the suckling and post-weaning fast, but also a similar change in other glucocorticoids cortisone and 11-deoxycorticosterone during the same period. Similar cortisol pattern was observed in saliva and plasma of healthy newborn human infants (Kiess et al., 1995; Tollenaar et al., 2010).

Higher concentrations of estradiol (by using both analysis methods – ELISA and RIA) and androstenedione in female pups were recorded for the first time in this study. Estrogens are related to immune system activity and cause sex-associated resistance to helminth parasite infection (Guzmán et al., 2009), while milk cortisol is associated with higher fat storage and body size in human newborn children (Pundir et al., 2020). Androstenedione is a steroidal hormone produced in male and female gonads, as well as in the adrenal glands, and it is known for its key role in the production of estrogen and testosterone (Badawy et al., 2021). Differences in steroid concentrations between nutritional categories and sex may thus have consequences for pups' behavior, immunity (Hall et al., 2002), energy balance (Bennett et al., 2013) and sensitivity to endocrine disrupting chemicals (Bäcklin et al., 2003; Troisi et al., 2020) and may contribute to reported sex differences in their survival (Hall et al., 2001).

2.5. CONCLUSIONS

1. Saliva sampling did not require a venipuncture, had a low risk of infection and short processing in the laboratory, but it required a longer collection time and yielded a lower collection success rate, therefore appropriate for collection of saliva from trained captive phocid seals.
2. Estradiol and cortisol assays performed well and can be used in plasma and saliva, even in young grey seal pups, however high variability of plasma estradiol concentrations requires further analysis. Testosterone could not be validated for plasma assays due to high inter-assay variability between the plates.
3. Saliva provided similar detail on estradiol and cortisol differences between nutritional states compared with plasma. However, females consistently had higher estradiol than males in the matched plasma samples, whereas no sex differences were observed in saliva, which might limit its utility for measuring sex steroids in young animals.
4. Plasma cortisol was highly comparable between two different methods – ELISA and UPC_2-MS/MS. Even though, estradiol concentrations between two different methods – ELISA and RIA – were positive, this relationship was not significant. It was impossible to compare testosterone concentrations between ELISA and RIA due to very low concentrations.
5. Strongest links were found between glucocorticoids and pup behavior. Corticosterone and 11-deoxycortisone were positively related with proportion of social behavior in grey seal pups, while 11-deoxycortisol was strongly linked to resting behavior.

3. GENERAL DISCUSSION

In the beginning of this study the description of social play that takes place during the nonbreeding season on land was provided, where young males predominated in the social activities. The social play during the suckling and post-weaning fast presented in the second chapter was almost nonexistent in grey seal pups, this contrast with other carnivore species in which youngsters start to play with each other soon after they start moving independently (Bekoff, 1974b; Burghardt, 2005; Drea et al., 1996; Pellis and Pellis, 1990). A similar tendency has been seen in otariid species in which pups begin social play during the suckling period (Gentry, 1974; Harcourt, 2010). The behavioral budget of grey seal suckling and weaned pups found in this study resembled those reported previously (Kovacs, 1987b), the highest proportion of behavioral budget consists of resting behavior. Suckling and mother pup interactions were the main social contacts, while no other than mother directed contacts were observed during suckling period. Non-social investigation was also the main nonsocial activity, as reported in a study mentioned above. Despite lower duration of observations during the post-weaning fast that might cause a high variability on the budget of the resting behavior, it still makes a large proportion of pup behavioral repertoire during the post-weaning fast. Differently from the suckling period, weaned pups were alert for around 40% of total observation time followed by 10 % of non-social investigation. Social identification per distance was the most abundant social behavior compared to the suckling period. Social contact behavior switched from almost 20 % of suckling and mother-pup interaction during suckling to less than 1 % with social investigation per contact and social play altogether. During fasting animals are saving energy and spend more time alone, which can be related to their aquatic lifestyle and energetic requirements on land. As mentioned previously, differently from the water, play on land requires more energetic recourses, because movements of true seals are more restricted on land (Garrett and Fish, 2015; Tennett et al., 2018). Also experience and possibility to predict the behavior of others might also play a role in pup lack of social contacts. The nature of mother-pup relationship is mostly limited to muzzling, sniffing, directing the pup to the nipple and suckling with rare moments of playful interactions probably because mothers themselves need to save energy. Pup experience towards social interactions with other conspecific are mostly of agonistic nature, in which adult females act aggressively towards

an unfamiliar pup (Robinson et al., 2017, 2015; Twiss et al., 2012a). Thus the motivation and possibility to interact socially during the suckling period is limited and this process appears to be shifted to the post-weaning fast period. On the contrary, pups raised in captivity and dependent on human behavior are much more social and playful probably due to a different human-seal nature of interactions (auth. pers. obs.), showing that lack of socialness is not an innate aspect of behavior, but rather a consequence of environmental conditions. According to author's observations in captivity, first contact between the pups takes place in the water and contact play on land starts after one month of interactions, meaning that they need time to familiarize to each other and the behavioral repertoire. This corresponds with Robinson et al. (2015) experimental trials with wild weaned grey seal pups in which familiar pups exhibited less checks and agonistic behaviors.

The most apparent strong and consistent positive links were observed between corticosterone (COS) and the proportion of social behaviors. Those pups that had on average higher concentrations of COS spent more time suckling (Skl) or in a close contact with their mothers (MP) during the suckling period. Similarly, weaned pups that had higher average COS levels spent more time for social investigation per distance (SI_D) and per contact (SI_C), as well as vocalization (Voc) during the post-weaning fast. Similar, but weaker positive correlations were seen between proportions of vocalization and social investigation per contact and the precursor of corticosterone – 11-deoxycorticosterone (DOC). Corticosterone is considered to be a main circulating glucocorticoid in rodents while cortisol is the dominant in other mammal species where corticosterone acts more like an aldosterone – active mineralocorticoid – precursor. However, our research might provide a new functional link between seemingly less potent glucocorticoid in grey seals and early social behaviors in grey seal pups. In marine mammals, as in other terrestrial mammals, corticosterone can be converted by blubber to cortisol and vice versa (Galligan et al., 2018a). Recent studies showed that corticosterone is very important for social maternal behavior and memory of rats after parturition (Graham et al., 2006; Rees et al., 2004). Higher doses of corticosterone administered to primiparous adrenalectomized mice after parturition increased levels of their maternal behavior. Corticosterone is not necessary for the initiation or maintenance of maternal behavior but plays a role in the modulation of ongoing maternal behavior, enhances maternal memory and initial maternal behavior in postpartum rats (Graham et al., 2006; Rees et al., 2004). In rat pups, corticosterone increases early-life stress

rates naturally or through the mother's milk (Yeh, 1984). Additionally, the mother modulates a pup's corticosterone concentration based on the level of her maternal care: a decrease in maternal care causes an increase in the pup's corticosterone levels (Stanton and Levine, 1988; Suchecki et al., 1995). Maternal presence reduces corticosterone concentrations and induces pup attachment (Moriceau and Sullivan, 2006). This study shows that those grey seal pups who have higher concentrations of corticosterone, seek for mother's presence via mother pup contact and suckling for food. Food seeking related timing of the departure from the colony was positively linked to corticosteroid concentrations in Northern elephant and Antarctic fur seals (Guinet et al., 2004; Ortiz et al., 2003). As artificial cortisol – dexamethasone failed to explain weaned grey seal departure from the colony (Bennett et al., 2013), other potential steroids like corticosterone could act as a trigger, because it signals the need or motivation to search for food.

In contrast, a positive behavior of another glucocorticoid, the precursor of cortisol – 11-deoxycortisol (11-deoxyCOR) and resting behavior (rest behavior – during suckling and comfort moves during resting – during post-weaning fast) were observed which could be linked to metabolic processes during resting activities. Though we did not include cortisol in behavioral analysis due to limited ability to control for stress effect during and prior capture, a strong link of less potent cortisol precursor and resting behavior was found. Cortisol itself is linked to regulation of the long term energy balance by enhancing the gluconeogenic capacity of the liver, facilitate mobilization of fat and proteins, and increase blood glucose levels during fasting (Akalestou et al., 2020; Bennett et al., 2013). The link of cortisol direct precursor and the proportion of rest behavior might indicate that during the resting activity metabolic and probably gluconeogenic activities take place, however it is hard to tell which activity induces the other. It is known that grey seal pups overcome extreme physiological changes during early development period, e. g. development of muscle tissue, formation of different brain and bone structures, changes in hematocrit, myoglobin levels by utilizing blubber fat (Noren et al., 2008, 2005; Worthy and Lavigne, 1987). The relationship between sleep and gluconeogenesis is well known from human studies, where lack of non-REM sleep reduces gluconeogenesis, lipolysis and increases risk for obesity (Briançon-Marjollet et al., 2015; Morselli et al., 2010; Zhang et al., 2021). In this case 11-deoxyCOR might be important for inducing sleep behavior for proper metabolic processes during critical suckling and post-weaning fast periods.

Differently from the hypothesis, there were no links between behavior and sex hormones testosterone and estradiol. Here we were also unable to look at sex differences in behavior due to a small female sample size. Neither concentrations of estradiol (EB_RIA), nor those of estrone (E1) had any link to behavior. However, estradiol might have some sex depended physiological roles previously discussed. These results show that sex steroids are detectable in quite large concentrations during early development when animals are immature, and might lead to sex-specific social behaviors already visible during grey seal adolescence.

Low levels of ELISA and RIA testosterone did not allow to make comparison with behavior, and those obtained with UPC_2-MS/MS were very low or below the LOQ/LOD as well, thus providing a weak model. Some other androgens showed significant correlations: androstenedione low negative correlation with non-social play while dehydroepiandrosterone had a good negative correlation with social investigation per distance; positive correlations were found between androstenediol and vocalization, dihydrotestosterone – with social investigation per contact and suckling behaviors. These links are difficult to investigate due to low concentrations (<LOD/LOQ) of mentioned steroids and very small values of Voc and SI_C as well, therefore making these links as artefacts of a small and low value sample size, and thus leading to poor statistical values. However, it provides grounds for further investigations in the future.

The overall study provides a description of social activities from early behavior to adolescence in grey seals, the differences of social repertoire in different developmental stages and sex specific differences of social play that can only be seen at later developmental stages closer to maturity. Several different steroid analysis methods allowed investigation of multiple steroids in grey seals during the suckling and post-weaning fast. Some of these steroids, like sex steroids and a big part of glucocorticoids were determined in suckling and weaned grey seals for the first time. A pilot study searching the links between steroid levels and behavior using correlation leaves room for interpretation that positive correlation of corticosterone and its precursor on social behaviors and 11-deoxycortisol on resting behavior, and might be explained by other factors that were not measured and evaluated here. Unfortunately, a small behavioral data set did not allow to use stronger modeling methods for determination of this link. However, the links presented in this research do match previous studies and provide a basis for future investigation of relationship between the early grey seal pup behavior and glucocorticoids.

3.1. CONCLUSIONS

1. The majority of interactions were performed by sub-adult males (61,76 %), followed by sub-adult females (13,53 %). The behavioral repertoire mainly consisted of play fight elements, mostly wrestling (14 %), which was frequently expressed during sub-adult male social play interactions.
2. Only group size predominated by males, availability of haul out space and time closer to the breeding season had a significant positive effect on the number of play interactions.
3. Saliva sampling did not require a venipuncture, had a low risk of infection and short processing in the laboratory, but it required a longer collection time and yielded a lower collection success rate, therefore appropriate for collection of saliva from trained captive phocid seals.
4. Estradiol and cortisol ELISA assays performed well and can be used both for plasma and saliva samples even in young grey seal pups; however, saliva samples provided lower resolution for estradiol than plasma samples. Plasma cortisol, but not estradiol, was highly comparable between two different steroid analysis methods.
5. Strongest links were found between glucocorticoids and pup behavior. Corticosterone positively related with proportion of social behavior in grey seal pups, while 11-deoxycortisol was positively linked to resting behavior.

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SUPPLEMENTARY MATERIAL

Table S1. Study reports on pinniped steroid concentrations in saliva and plasma. *LC-MS* – liquid chromatography mass spectrometry, *LC-MS/MS* – liquid chromatography tandem mass spectrometry, *RIA* – radioimmunoassay, *CLIA* – chemiluminescence immunoassay, *ECL* – electro chemiluminescence immunoassay, *EIA* – enzyme immunoassay, *ELISA* – enzyme linked immunosorbent assay, *GnRH* – gonadotropin releasing hormone.

Hormone	Matrix	Concentration	Collection	Method	Extraction	Animal	Age	Author
Testosterone	Saliva	30-190 pg/ml	Fibrous absorbent material used for filters	RIA	Diethyl ether	Hawaiian monk Seal	Adult males	(Theodorou and Atkinson, 1998)
	Saliva	0,3-1,2 ng/ml (~1 nmol/l)		RIA	No	Stellar sea lions	Adult males	(Harmon, 2001)
	Blood	~ 0,29 ng/ml	Heparin plasma	RIA	No	Harbor seals	< 4 years old	(Lydersen and Kovacs, 2005)
		~ 1,73 ng/ml (~ 6 nmol/l)					>7 years old	
	Blood	From 0,09 ng/ml to 1,78 – 4,04 ng/ml	Not clear	RIA	Diethyl ether	Monk seals	Adults	(Atkinson and Gilmarti, 1992; Theodorou and Atkinson, 1998)
	Blood	0,01 ng/ml up to 9,4 ng/ml	Heparin plasma	RIA	No	Harp seals	Adult captive	(Serrano, 2000)
	Blood	1-2 ng/ml up to 20-25 ng/ml	Plasma	RIA	1:10 Ether : hexane (1:1)	Southern elephant seal	Adults	(Griffiths, 1984)
	Blood	6,8 ng/ml	Serum	RIA	No	Weddell seals	Adult males, breeding season	(Bartsh et al., 1992)
	Blood	0,01 ng					Adult males, non-breeding season	
	Blood	0,2-0,9 ng/ml	Plasma	RIA	No	Steller sea lion	Adult males	(Harmon, 2001)
	Blood	6,2 ng/ml up to 8 ng/ml	Serum	CLIA	No	Grey seal	Adult males (17-34 years old) during breeding season	(Lidgard et al., 2008)

Hormone	Matrix	Concentration	Collection	Method	Extraction	Animal	Age	Author
Testosterone	Blood	Up to 6 nmol/l (1,73 ng/ml)	Heparin plasma	RIA	No	Harbor seals	Adults and juveniles	(Gardiner et al., 1999)
	Blood	3,2 nmol/l (0,92 ng/ml)	Serum	RIA	No	Southern Elephant seals	Adult males	(Ferreira et al., 2005)
		0,4-1,1 nmol/l (0,15-0,32 ng/ml)					Weaners – juveniles	
	Blood	1,01-0,21 ng/ml treated group, 1,11-1,16 ng/ml) untreated	Circulating	RIA	Diethyl ether	Hawaiian Monk seals	Wild adults males	(Atkinson et al., 1998)
	Blood	10,81 ± 9,57 nmol/L peak, 1,42 ± 3,09 nmol/L	Serum	ECL	No	Spotted seals	> 4 years old captive females	(Zhang et al., 2014)
	Blood	0,02-2,18 ng/ml (undetected after buserelin acetate 9,9 mg)	Serum	LC-MS/MS	N/A	Harbor seals	Captive adults males	(Siebert et al., 2007)
	Blood	5,63-14,31 nmol/l (1,62-4,13 ng/ml)	Heparin plasma	RIA	No	Weddell seals	Adult males, early – late breeding season	(Harcourt et al., 2010)
	Blood	~40 ng/g	Sodium-heparin plasma	LC-MS	SPE	Bottlenose dolphins	Adults	(Galligan et al., 2018a)
		~1 ng/g					Juveniles	
Estradiol	Blood	~10 pmol/l (2,72 pg/ml)	Heparin plasma	RIA	No	Harbor seals	< 4 years old	(Lydersen and Kovacs, 2005)
		> 20 pmol/l (5.44 pg/ml)					> 4 years old	
	Blood	15,80 ± 14,15 ng/L peak, 7,77 ± 6,78 ng/L low	Serum	ECL	No	Spotted seals	Older than 4 years captive females	(Zhang et al., 2014)

Hormone	Matrix	Concentration	Collection	Method	Extraction	Animal	Age	Author
Estradiol	Blood	30 pg/ml	N/A	N/A	N/A	Northern fur seals	N/A	(Dierauf and Gulland, 2001)
	Blood	Up to 400 pmol/l (108,95 pg/ml)	Serum	RIA	No	Harbor seals	Adults	(Reijnders, 1998)
		Up to 120 pmol/l (32,68 pg/ml)					Immatures	
	Blood	188 pmol/l (51,21 pg/ml)	Serum	RIA	No	Southern Elephant seals	Adult females	(Ferreira et al., 2005)
		~100 pmol/l (27,24)					Weaners and juveniles	
	Blood (Estrone suphate)	0,1 – 10 ng/ml	Plasma	RIA	No	Hawaiian monk seals	Adult captive females	(Pietraszek and Atkinson, 1994)
	Saliva (Estrone sulphate)	0 -10 ng/ml	Fibrous material/ sponge	RIA	No	Hawaiian monk seals	Adult captive females	(Pietraszek and Atkinson, 1994)
Cortisol	Blood	164-215 nmol/l (59,45-77,94 ng/ml)	Serum	RIA	No	Southern Elephant seals	Adult males and females	(Ferreira et al., 2005)
		153-273 nmol/l (55,46-98,96 ng/ml)					Juveniles-weaners	
	Blood	50-120 ug/dl (ng/ml)	Serum	RIA	No	Weddell seals	Adult males at breeding season	(Bartsh et al., 1992)
	Blood	9,7 ug/dl (97 ng/ml) up to 17 ug/dl (170 ng/ml)	Serum	CLIA	No	Grey seals	Adult (17-34 years old) males, breeding season	(Lidgard et al., 2008)
	Blood	60 nmol/l (21,9 ng/ml) to 100 nmol/l (37,03 ng/ml)	Serum	RIA	No	Grey seals	Pups and weaners	(Bennett et al., 2012)
	Blood	1000-1600 nmol/l (362,5-580 ng/ml)	Heparin plasma	RIA	No	Weddell seals	Adult males	(Harcourt et al., 2010)
	Saliva	1-40 ng/ml (around 5-8 ng/ml)	Saliva	ELISA	No	Antarctic fur seals	Adult females and pups	(Nagel et al., 2022)

Hormone	Matrix	Concentration	Collection	Method	Extraction	Animal	Age	Author
Aldosterone	Blood	217-260 pg/ml (602,6 -721,6 pmol/l)	Serum	RIA	No	Southern Elephant seals	Adult females and males	(Ferreira et al., 2005)
		1056,0 pmol/l (380,6 pg/ml)					Weaned	
Progesterone	Blood	0,58 ± 0,1 ng/ml to 8,83 ± 4,2 ng/ml	Plasma	RIA	Diethyl ether	Hawaiian monk seals	Adult captive females	(Pietraszek and Atkinson, 1994)
	Blood	9-63 ng/ml (30-250 nmol/ml)	Plasma	ELISA	N/A	Harbour seals	Females	(Gardiner et al., 1999)
	Saliva	0 – 12 ng/ml	Fibrous material/ sponge	RIA	Washed out with buffer	Hawaiian monk seals	Adult captive females	(Pietraszek and Atkinson, 1994)
17a-OHP	Blood	12,4 ng/ml (37,39 ± 17,03 nmol/L) peak, 0,24 ng/ml (0,74 ± 0,54 nmol/L) low	Serum	ECL	No	Spotted seals	> 4 years old captive females	(Zhang et al., 2014)
	Blood	6 ng/ml during gestation, 10 ng/ml during final month, <1 ng/ml at parturition	Plasma	RIA	N/A	Grey seal	Wild adult females	(Boyd, 1984)

Table S2. ID, sex and sampling regime of grey seal pups whose samples were included in the analysis together with analysis specifications (plate number (year of sample analysis)) are provided in the table below. Terms *No saliva collected* indicates that we failed to collect minimum amount of 100 µl due to low saliva productivity or the sample was contaminated with blood or other substances; *Not anesthetized* – animal was not injected with anesthetics during handling; *Not caught* – animal not caught for the research due to specific research purposes or when not found in the colony for the last catch; *Pup age stages* – 2, 3, 4, 5 according to Kovacs and Lavigne (1986) and *exact age (days)*, based on date of birth or calculations from the body mass and age stage (Pomeroy et al., 1999) is also presented for every individual.

Nr.	Collection year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
1	2012	45424	M	Blood not analysed/ lack of sample Not anesthetized Stage – 2 Age – 8	EB, TB, CB (*Plate II [2015]*) Not anesthetized Stage – 4 Age – 16	Not caught	Not caught
2	2012	45251	M	EB, TB, CB (*Plate II [2015]*) CS, ES (lack of sample), **TS (above the limit)** Stage – 2 Age – 8	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 4 Age – 15	EB, TB, CB, (*Plate I [2014]*) ES, TS, **CS** Stage – 5 Age – 27	Not caught
3	2012	45410	M	EB, TB, CB, (*Plate I [2014]*) ES, CS, **TS (above the limit)** Stage – 3 Age – 10	EB, TB, CB, (*Plate I [2014]*) ES, CS, TS Stage – 4 Age – 18	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 5 Age – 27	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 37
4	2012	51115	M	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 2 Age – 7	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 4 Age – 18	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 5 Age – 26	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 36

Nr.	Collec-tion year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
5	2012	54015	F	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 2 Age – 8	EB, TB, CB Both plates: Inter-CV%: EB: 3,15 % TB: 73,68 % CB: 29,76 % No saliva collected Stage – 4 Age – 16	Not caught	Not caught
6	2012	57024	M	EB, TB, CB (*Plate II [2015]*) Not anesthetized Stage – 2 Age – 8	Not caught	Not caught	Not caught
7	2012	57045	F	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 3 Age – 10	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 4 Age – 17	EB, TB, CB (*Plate I [2014]*) CS, TS (lack of sample), ES (lack of sample) Stage – 5 Age – 27	Blood not analyzed/ lack of sample. ES, TS, CS Stage – 5 Age – 37
8	2012	57062	F	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 3 Age – 9	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 4 Age – 17	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 28	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 38
9	2012	58038	F	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 2 Age – 6	EB, TB, CB, (*Plate I [2014]*) ES, CS, **TS (above the limit)** Stage – 4 Age – 17	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 28	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 38

Nr.	Collec-tion year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
10	2012	58785	F	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 2 Age – 6	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 4 Age – 16	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 23	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 33
11	2012	72146	M	EB, TB, CB (*Plate II [2015]*) TS, CS, ES (lack of sample) Stage – 2 Age – 5	EB, TB, CB, (*Plate I [2014]*) No saliva collected Stage – 4 Age – 15	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 26	EB, TB, CB, (*Plate I [2014]*) TS, CS, ES (lack of sample) Stage – 5 Age – 37
12	2012	1W	F	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 2 Age – 9	Not caught	Not caught	Not caught
13	2012	2W	M	CS, ES (lack of sample), TS (lack of sample) Blood not analysed/ lack of sample Stage – 2 Age – 4	Not caught	Not caught	Not caught
14	2012	3U	F	EB, TB, CB, (*Plate I [2014]*) TS, CS, ES (lack of sample) Stage – 2 Age – 5	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 5 Age – 16	EB, TB, (*Plate II [2015]*) CS, TS (lack of sample), ES (lack of sample) Stage – 5 Age – 21	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 33

Nr.	Collec-tion year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
15	2012	4H	M	EB, TB, CB (*Plate II [2015]*) Not anesthetized Stage – 2 Age – 6	EB, TB, CB (*Plate II [2015]*) Not anesthetized Stage – 4 Age – 15	Not caught	Not caught
16	2012	4B	M	ES, TS, CS (lack of sample) Blood not analysed/ lack of sample Stage – 2 Age – 6	No saliva collected Blood not analysed/ lack of sample Stage – 4 Age – 16	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 4 Age – 26	Not caught
17	2012	6J	F	EB, TB, CB Both plates: Inter-CV%: EB – 13,83 %, TB – 98,81%, CB – 20,68% No saliva collected Stage – 2 Age – 6	Blood not analysed/ lack of sample No saliva collected Stage – 4 Age – 17	Not caught	Not caught
18	2012	72448/9	M	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 2 Age – 5	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 4 Age – 15	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 26	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 36
19	2012	7J	M	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 2 Age – 6	Blood not analysed/ lack of sample No saliva collected Stage – 4 Age – 17	Blood not analysed/ lack of sample No saliva collected Stage – 5 Age – 25	Blood not analysed/ lack of sample ES, TS, CS Stage – 5 Age – 35
20	2012	8N	M	EB, TB, CB (*Plate I [2014]*) Not anesthetized Stage – 2 Age – 7	Not caught	Not caught	Not caught

Nr.	Collection year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
21	2012	D8	M	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 2 Age – 6	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 4 Age – 17	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 5 Age – 26	Not caught
22	2012	0H	F	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 2 Age – 4	EB, TB, CB, (*Plate I [2014]*) ES, TS, CS Stage – 4 Age – 17	Not caught	Not caught
23	2012	0J	F	Blood not analysed/ lack of sample No saliva collected Stage – 2 Age – 4	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 4 Age – 15	EB, TB, CB (*Plate II [2015]*) ES, TS, CS Stage – 5 Age – 25	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 5 Age – 35
24	2012	PFT	F	EB, TB, CB (*Plate I [2014]*) No saliva collected Stage – 2 Age – 6	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 5 Age – 17	EB, TB, CB (*Plate II [2015]*) No saliva collected Stage – 5 Age – 25	Blood not analysed/ lack of sample ES, TS, CS Stage – 5 Age – 35
25	2017	OJ	F	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 2 Age – 6	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 4 Age – 16	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 25	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 37

Nr.	Collec-tion year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
26	2017	58038	M	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 2 Age – 6	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 4 Age – 16	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 23	EB (<LOD), TB(<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 35
27	2017	6J	M	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 2 Age – 8	Not caught	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 29	Not caught
28	2017	72053	M	Not caught	EB_RIA UPC_2-MS/MS No saliva collected Stage – 3 Age – 8	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 4 Age – 20	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 29
29	2017	72159	F	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 2 Age – 5	EB, TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 4 Age – 15	EB, TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 28	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 34

Nr.	Collection year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
30	2017	74323	M	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 2 Age – 5	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 4 Age – 15	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES (Not enough sample), TS, CS Stage – 5 Age – 24	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 36
31	2017	74790	M	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 2 Age – 8	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 4 Age – 18	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 24	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), ES, TS, CS Stage – 5 Age – 37
32	2017	74890	M	EB, TB, CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 2 Age – 3	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 4 Age – 15	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 27	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 5 Age – 37
33	2017	75334	F	EB, TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS No saliva collected Stage – 2 Age – 5	Blood not analysed/ lack of sample No saliva collected Stage – 4 Age – 15	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, **CS** Stage – 5 Age – 29	EB (<LOD), TB (<LOD), CB (*Plate IV [2018]*), EB_RIA UPC_2-MS/MS ES, TS, CS Stage – 5 Age – 35

Nr.	Collection year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
34	2016	43557	M	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 6	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 18	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 23	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 40
35	2016	45424	M	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 4	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 15	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 21	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 37
36	2016	59074	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 3	Not caught	Not caught	Not caught
37	2016	72053	M	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 3	Not caught	Not caught	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 38
38	2016	72146	M	EB, TB, (*Plate III [2018]*), No saliva collected Stage – 2 Age – 5	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 15	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 20	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 36
39	2016	74695	M	EB, TB, (*Plate III [2018]*), No saliva collected Stage – 2 Age – 2	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 15	Not caught	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 32

Nr.	Collec-tion year	Animal ID	Sex	Early suckling (S1) 4-10 days old	Late suckling (S2) 15-18 days old	Early post weaning (W1) 21-28 days old	Late post weaning (W2) 33-38 days old
40	2016	74916	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 6	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 16	EB (<LOD), TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 28	Not caught
41	2016	75331	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 3	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 13	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 23	Not caught
42	2016	75334	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 4	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 4 Age – 15	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 27	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 5 Age – 32
43	2016	6L	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 3	Not caught	Not caught	Not caught
44	2016	74962/3	F	EB, TB (<LOD), (*Plate III [2018]*), No saliva collected Stage – 2 Age – 5	Not caught	Not caught	Not caught

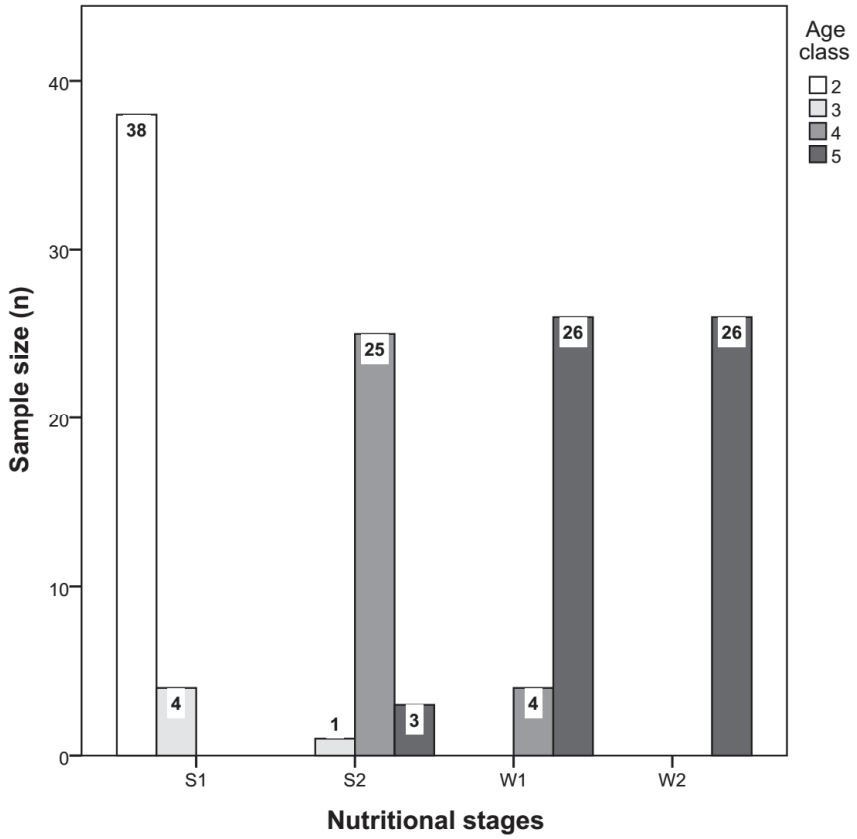

Fig. S1. The frequency (n = 122) of sampled pups from the Isle of May (2012, 2016, 2017) in different age stages (2-5) during different nutritional stages (S1 – W2).

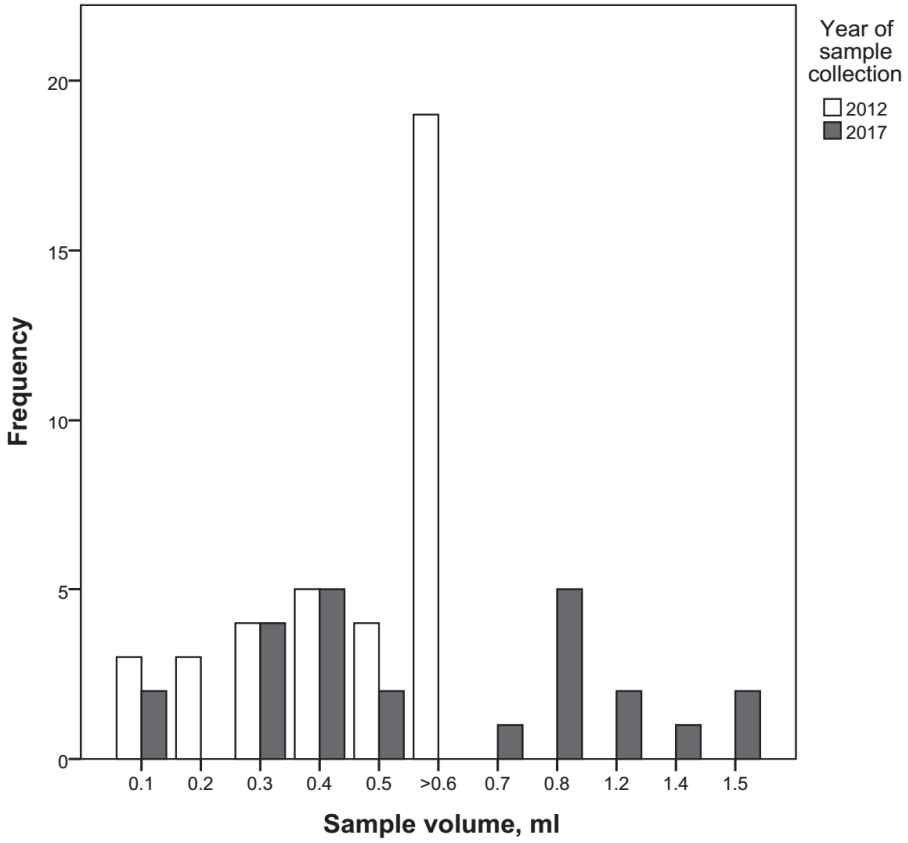

Fig. S2. The frequency (n = 62) of saliva volume (ml) of individual samples from different sampling years (2012 and 2017).

Table S3. Name, abbreviation, molecular formula and multiple reaction monitoring parameters for steroid hormones in UPC2-MS/MS analysis. *IS* internal standard, *RT* retention time, *CV* cone voltage, *CE* collision energy. [#] indicates quantifier ion, [a] neutral loss of H_2O, [b] neutral loss of H_2O.

Steroid Class	Name, Abbreviation (IS)	Molecular Formula	Transitions	RT	CV (V)	CE (eV)
Androgens	Dehydroepiandrosterone, DHEA (DHT $^{13}C_3$)	$C_{19}H_{28}O_2$	253 > 197[#b] 253 > 213[b]	1.45	20 20	18 18
	Androstenedione, AN (DHT $^{13}C_3$)	C19H26O2	287 > 97[#] 287 > 109	1.59	20 20	22 26
	Androstenediol, A5 (DHT $^{13}C_3$)	C19H30O2	255 > 159[#b] 255 > 145[b]	1.41	20 20	18 18
	Testosterone, TS (DHT $^{13}C_3$)	C19H28O2	289 > 97[#] 289 > 109	1.94	20 20	20 26
	5α-Dihydrotestosterone, DHT (DHT $^{13}C_3$)	C19H30O2	291 > 159[#] 291 > 255	1.40	24 24	24 16
	11-Ketotestosterone, KetoTS (CORNE $^{13}C_3$)	C19H26O3	303 > 121[#] 303 > 267	2.24	18 18	26 18
Corticosteroids	11-deoxycorticosterone, DOC (17aOHP_13C3)	C21H30O3	331 > 97[#] 331 > 109	1.72	20 20	20 22
	11-deoxycortisol, 11-deoxyCOR (CORNE $^{13}C_3$)	C21H30O4	347 > 347[#] 347 > 97	2.16	20 20	32 10
	Aldosterone, ALDO (CORNE $^{13}C_3$)	C21H28O5	361 > 315[#] 361 > 325	2.50	20 20	20 18
	Corticosterone, COS (CORNE $^{13}C_3$)	C21H28O5	347 > 91[#] 347 > 97	2.34	20 20	46 22
	Cortisol, COR (CORNE $^{13}C_3$)	C21H30O5	363 > 121[#] 363 > 327	2.59	20 20	24 14
	Cortisone, CORNE (CORNE $^{13}C_3$)	C21H28O5	361 > 121[#] 361 > 163	2.25	20 20	30 24
Progestogens	Pregnenolone, PREG (DHT_13C3)	C21H32O2	299 > 159[#a] 299 > 281[a]	1.43	20 20	20 20
	Progesterone, P4 (DHT_13C3)	C21H30O2	315 > 97[#] 315 > 109	1.56	34 34	20 24
	17a-hydroxyprogesterone, 17α-OHP (17aOHP_13C3)	C21H30O3	331 > 97[#] 331 > 109	1.90	20 20	26 24
Estrogens	Estrone, E1 (17aOHP_13C3)	C18H22O2	271 > 133[#] 271 > 197	1.68	22 22	35 35

Internal Standards						
Corticoste-roids	Cortisone-13C3, 2,3,4-13C3-CORNE	C18*C3H28O5	364 > 123[#] 364 > 166	2.25	20 20	30 24
Androgens	Dihyrdotestosterone-13C3, 2,3,4-13C-DHT	C16*C3H30O2	294 > 162[#] 294 > 258	1.40	24 24	24 16
Progesto-gens	17α-hydroxyprogesterone-13C2, 2,3,4-13C2-17α-OHP	C18*C3H30O3	333 > 99[#] 333 > 111	1.90	20 20	26 24

LIST OF PUBLICATIONS

Publications in journals with impact factor included in *Clarative Analytics Web of Science* database:

Survilienė, V., Rukšėnas, O., Pomeroy, P. P., Moss, S. E. W. & Bennett, K. A. 2022. Evaluating suitability of saliva to measure steroid concentrations in grey seal pups. General and Comparative Endocrinology, 326, 114070. https://doi.org/10.1016/j.ygcen.2022.114070

Survilienė, V., Rukšėnas, O. & Pomeroy, P. P. 2016. Play behavior of wild Grey seals: Effects of haul-out group size and composition. Aquatic Mammals, 42(2), 144-161. https://doi.org/10.1578/AM.42.2.2016.144

Saint, S. T. L., *Survilienė, V.*, Jüssi, M., Gonzalez, S. V., Ciesielski, T. M., Jenssen, B. M. & Asimakopoulos, A. 2022. Determination of steroid hormones in grey seal (*Halichoerus grypus*) blood plasma using convergence chromatography tandem mass spectrometry. Talanta, in Press: https://doi.org/10.1016/j.talanta.2022.124109

International conferences attended:

Survilienė, V., Sait, S.T.L, Asimakopoulos, A. G., Bennet, K., Moss, S., Pomeroy, P., Rukšėnas, O., Jenssen, B.M. & Ciesielski, T. M. 2022. Steroid hormone profiles in grey seal pups during the suckling period and postweaning fast using SFC-MS/MS. 24th Biennial Conference on the Biology of Marine Mammals, rugpjūčio 1-5, Florida, USA.

Survilienė, V., Moss, S. & Rukšėnas, O. Changes in saliva steroid hormone levels of grey seal pups during lactation and post-weaning fast. World Marine Mammal Conference, gruodžio 9-12, 2019, Barcelona, Spain.

Stukonytė, L., Pomeroy, P., Twiss, S., Mozgeris, G., Rukšėnas, O. & *Survilienė, V*. Evidence of group density effect on behavioural differences in grey seal (*Halichoerus grypus*) neonates during lactation period. World Marine Mammal Conference, gruodžio 9-12, 2019, Barcelona, Spain.

Survilienė V., Pomeroy P., Moss, S. & Rukšėnas O. The comparative analysis of steroid hormones in saliva and blood from grey seal (*Halichoerus grypus*) pups. 22nd Biennial Conference on the Biology of Marine Mammals, spalio 22-27, 2017, Halifax, Canada.

Survilienė, V., Moss S., Pomeroy P. & Rukšėnas O. The analysis of steroid hormones in saliva and blood from grey seal (*Halichoerus grypus*) pups. 20th Anniversary Conference Laboratory Animals in Research, lapkričio 24-25, 2016, Vilnius, Lietuva.

Survilienė, V., Pomeroy, P., Moss S. & Rukšėnas, O. The use of saliva samples to estimate levels of steroids in grey seal (*Halichoerus grypus*) pups. Conference of Life Sciences Baltic, Vilnius, 2016.

CURRICULUM VITAE

Education:

- 2013-2022 – PhD in Biology, Vilnius University.
- 2010-2012 – MSc in Zoology, Vilnius University.
- 2006-2010 – BSc in Biology, Vilnius University.

Work experience:

- 2013 to present – junior research fellow/ junior assistant, Vilnius University.
- 2014-2015, 2019-2020 project manager, Lithuanian Fund for Nature.

Grants received:

- 2014 – grant for equipment purchase, IDEA WILD (http://www.ideawild.org/).
- 2013 - Society for Marine Mammalogy Grants in Aid of Research.

Expeditions/ internships:

- 2020 – internship at Norwegian University of Science and Technology, Norway.
- 2020 – Baltic grey seal pilot studies, Saarema, Estonia.
- 2009 – 2017 – behavioural and physiological data collection from grey seals, Isle of May, Scotland, UK.
- 2009 – grey seal behavioural data collection during the non-breeding season, Erasmus practice, Scotland, UK.

Supervision of Bachelor thesis:

- Dikaitė, G. 2022. Factors Affecting the movement speed and tendency to aggregate in weaned grey seal (*Halichoerus grypus*) pups during the post-weaning fast, 38 pg.
- Ricart, J. O. 2022. The development of early social behavior of grey seals (*Halichoerus grypus*), 44 pg.
- Valaitis, L. 2021. Group density effect during the lactation period on the development of social associations in grey seal (*Halichoerus grypus*) pups, 50 pg.

- Jarmontovičiūtė, G. 2020. Determination of the frequency of grey seal visits to the Lithuanian coast and the impact on fishing efficiency based on fishing logbook data, 79 pg.
- Stukonytė, L. 2019. Factors affecting the behavior of grey seal (*Halichoerus grypus*) pups during the lactation period, 57 pg.
- Jasaitė, E. 2019. Factors affecting the development of grey seal (*Halichoerus grypus*) behavior during the post-weaning fast, 46 pg.

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