Aus dem Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

# Validierung und Evaluierung einer Alternativen Probenentnahmetechnik zur Corticosteronbestimmung in Federn

**Inaugural-Dissertation**
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
**Marielu Voit**
Tierärztin aus Erlangen

Berlin 2022
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*Für meine Familie*

**Gendersensible Schreibweise dieser Arbeit**

Für die gendersensible Schreibweise von Texten dieser Arbeit, die sich auf den heutigen Standpunkt beziehen, wird das generische Maskulinum verwendet, das die neutrale Form sowie die männliche als auch weibliche Form eines Wortes einbezieht. Eingeschlossen werden in diese Schreibweisen auch nicht binäre Personen und Personen weiterer Geschlechter.

**Inhaltsverzeichnis**

**Abkürzungen**

| | |
|---|---|
| 3-R-Prinzip | deutsch auch „3-V-Prinzip“; Replacement (Vermeidung), Reduction (Verringerung), Refinement (Verfeinerung) |
| ACTH | Adrenocorticotropes Hormon |
| CAPS | Captive Animals' Protection Society |
| CORT | Corticosteron |
| CORTf | Federcorticosteron |
| CRH | Corticotropin-releasing Hormon |
| EAZA | European Association of Zoos and Aquaria |
| ELISA | Enzyme-linked Immunosorbent Assay |
| GC | Glucocorticoide |
| HPA-Achse | englisch: hypothalamic-pituitary-adrenal axis; deutsch: Hypothalamus-Hypophysen-Nebennierenrinden-Achse |
| PETA e.V. | People for the Ethical Treatment of Animals e.V. |
| TierSchG | Tierschutzgesetz |
| TierSchVersV | Tierschutz-Versuchstierordnung |
| TVT e.V. | Tierärztliche Vereinigung für Tierschutz e.V. |
| VdZ e.V. | Verband der Zoologischen Gärten e.V. |
| WAZA | World Association of Zoos and Aquariums |

# 1 Einleitung

Zoologische Gärten sind bestrebt die Lebensbedingungen für gehaltene Tiere optimal zu gestalten, um deren psychischen und physischen Bedürfnissen gerecht zu werden, und sicherstellen, dass neue wissenschaftliche Erkenntnisse dazu schnell umgesetzt werden (WAZA 2003). Um das Wohlbefinden von in Menschenobhut gehaltenen Vögeln objektiv beurteilen zu können, werden in Studien nicht nur Verhaltensbeobachtungen vorgenommen, sondern diese häufig mit der Messung von Corticosteron (CORT) kombiniert. Dieses zählt zu den Hormonen, die an der unmittelbaren Antwort auf Stressoren beteiligt sind (Sapolsky et al. 2000). CORT dient demnach in wissenschaftlichen Studien als nützlicher physiologischer Indikator, der auf Stressstimuli (Eu- oder Distress) reagiert (Harvey et al. 1980). Das Hormon CORT kann in unterschiedlichen Matrices gemessen werden: in Blut (Dehnhard et al. 2003;Dufty Jr 2008), Kot (Kotrschal et al. 2000;Dehnhard et al. 2003;Möstl et al. 2005), Eiern (Downing und Bryden 2008;Royo et al. 2008), Speichel (Hahn et al. 2011) oder Federn (Bortolotti et al. 2008;Romero und Fairhurst 2016). Welches Medium zur Bestimmung von CORT sinnvoll ist, steht in Abhängigkeit von der Fragestellung der Studie.

Hintergrund dieser Arbeit ist die wissenschaftliche Untersuchung des Wohlbefindens von in zoologischen Einrichtungen gehaltenen Vögeln mit unterschiedlichem Flugstatus (Reese et al. 2020a;Reese et al. 2020b;Haase et al. 2021). Die Matrix der Wahl zur Bestimmung von CORT ist hierbei die Feder. Die Messung von Federcorticosteron (CORTf) ermöglicht die spätere Rekonstruktion der Hormonausschüttung über den relativ langen Zeitraum des Federwachstums (Bortolotti et al. 2008;Romero und Fairhurst 2016). Zur Bestimmung von CORTf werden Federn von lebenden oder toten Vögeln, welche direkt am Körper entnommen werden, verwendet, ebenso wie ausgefallene (Bortolotti et al. 2008;Fairhurst et al. 2011;Lattin et al. 2011;Kennedy et al. 2013). Findet die Probenentnahme am lebenden Tier statt, ist der bisherige Standard die Feder mittels Rupfens zu sammeln. Hierbei ist jedoch anzunehmen, dass diese Technik Schmerzen beim Individuum verursacht (Gentle 1992;Gentle und Hunter 1991;Gentle et al. 1990;Malik und Valentine 2018). Deshalb ist für diese Art der Probenentnahme für eine wissenschaftliche Fragestellung die Beantragung eines Tierversuchs gesetzlich vorgeschrieben. Gleichzeitig sind Wissenschaftler nach dem EU Recht (Directive 2010/63/EU) und dem deutschen Recht (TierSchG und TierSchVersV) dazu verpflichtet, im Sinne des Tierwohls dem 3-R-Prinzip folgend zu handeln (Russell et al. 1959) und Methoden, wo immer möglich, zu verfeinern und zu verbessern. Daher befasst sich diese Arbeit mit der Validierung einer alternativen und weniger invasiven Federentnahmemethode: Die Feder wird nahe an der Haut abgeschnitten (Voit et al. 2020;Voit et al. 2021). Die Hypothese der vorliegenden Arbeit ist, dass die schonendere Entnahmemethode des

Federschneidens zu vergleichbar validen Aussagen in Bezug auf die gemessenen CORTf-Spiegel führt, wie der bisherige Standard des Federrupfens.

Die beiden Publikationen der vorliegenden Arbeit vergleichen CORTf Werte von gerupften und geschnittenen Federn mit verschiedenen statistischen Methoden. Hierbei werden an jedem Individuum beide Entnahmetechniken angewendet. Die erste Studie umfasst die Untersuchung von Hausgänsen (*Anser anser*) und Mulardenenten (*Anas sterilis* bzw. *Cairina moschata* × *Anas platyrhynchos*), die aus einer konventionellen Freilandhaltung stammen. In der zweiten Publikation wird CORTf von in der Wildbahn lebenden Rosaflamingos (*Phoenicopterus roseus*) und Stockenten (*Anas platyrhynchos*) analysiert.

# 2 Literatur

## 2.1 Hintergrund der Studie

### 2.1.1 Die Haltung von flugunfähigen Vögeln in zoologischen Einrichtungen

Neben geschlossenen Gehegen wie Volieren und Ökosystemhallen ist das Flugunfähigmachen von Vögeln in Außengehegen eine Möglichkeit der Haltung von Vögeln in zoologischen Gärten. Letzteres wird weltweit in Zoos an bestimmten Vogelarten angewendet und dient dem Zweck die Individuen an der Flucht aus meist offenen Freianlagen zu hindern (Dollinger et al. 2013). Grundsätzlich gibt es zwei Kategorien des Flugunfähigmachens: irreversible und reversible Methoden (Hesterman et al. 2001). Durch das steigende öffentliche Bewusstsein für Tierschutz und gesetzliche Änderungen stehen diese Methoden zunehmend in Kritik. Wird ein Blick auf §6 des Deutschen Tierschutzgesetzes geworfen, so herrscht in Deutschland ein Verbot der vollständigen oder teilweisen Amputation von Körperteilen oder der vollständigen oder teilweisen Entfernung oder Zerstörung von Organen oder Geweben eines Wirbeltieres (Tierschutzgesetz 1972). Mit der Abwandlung dieses Paragrafen 1998 wurde die Ausnahme "es sei denn, die Amputation ist für die Tierhaltung erforderlich" herausgenommen. Infolgedessen beschreiben einige Autoren die Praktiken des Flugunfähigmachens als eine Verletzung des Tierschutzes (Caps 2013;Bračko und King 2014;Tyson 2014;PETA 2017). Andere jedoch diskutieren diese Methoden als Möglichkeit bestimmte Vogelarten, die weniger vom Fliegen abhängig sind, auf eine bessere Weise, beispielsweise in größeren Freilandgehegen, halten zu können (Dollinger et al. 2013;Baumgartner 2015). Reese et al. (2020a) beschäftigen sich in ihrer Publikation mit den unterschiedlichen Techniken des Flugunfähigmachens, verbinden diese zusätzlich mit potenziellen ethologischen und tierschutzrelevanten Bedenken und zeigen darüber hinaus die gesetzlichen Regelungen in verschiedenen Ländern auf.

### 2.1.2 Studien zum Wohlbefinden flugunfähiger Vögel im Zoo

Die Aufgaben von zoologischen Einrichtungen definiert die EU-Zoorichtlinie 1999/22/EG: Erhaltung, Bildung und Forschung. Demzufolge sind die jeweiligen Tierarten bezüglich ihrer individuellen Bedürfnisse und ihres Wohlergehens zu schützen und zusätzlich das Entweichen der Tiere zu verhindern. Sowohl internationale Verbände, wie die World Association of Zoos and Aquariums (WAZA) oder die European Association of Zoos and Aquaria (EAZA), als auch nationale Verbände, wie die Deutsche Tierärztliche Vereinigung für Tierschutz e.V. (TVT),

fördern ethologische und physiologische Forschung zu verschiedenen Haltungsformen und streben einen gemeinsamen wissenschaftlich basierten Konsens zu flugunfähig gehaltenen Vögeln an (WAZA 2003;EAZA 2014;TVT 2015). Die TVT führt des Weiteren in einer Stellungnahme eine Liste an Vogelarten auf, bei denen das temporäre Flugunfähigmachen durch Beschneiden der Schwungfedern tierschutzrechtlich vertretbar ist (TVT 2015). Die Bewertung des Wohlbefindens dieser aufgeführten Arten sollte jedoch bezüglich des Flugstatus mit wissenschaftlich tierbasierten Untersuchungen evaluiert werden. Die Liste beinhaltet folgende Arten:

- Flamingos aus der Ordnung Phoenicopteriformes
- Pelikane aus der Gattung *Pelecanus* ssp. außer Rötelpelikan (*P. rufescens*) und Graupelikan (*P. philippensis*)
- Gänsevögel aus der Ordnung Anseriformes außer Pfeifgänse der Gattung *Dendrocygna* ssp., Zwergglanzgänse der Gattung *Nettapus* ssp., Rotschulterente (*Callonetta leucophrys*), Sturzbachente (*Merganetta armata*), Hartlaubente (*Pteronetta hartlaubi*), Weißflügelente (*Asarcornis scutulata*) und Spaltfußgans (*Anseranas semipalmata*)
- Kraniche aus der Familie Gruidae
- Lappentaucher und Seetaucher aus der Ordnung Podicipediformes und Gaviiformes
- Große Trappen aus den Gattungen *Otis* ssp. und *Ardeotis* ssp.
- Hornraben aus der Gattung *Bucorvus* ssp.
- Marabu (*Leptoptilos crumeniferus*) und Weißstorch (*Ciconia ciconia*)

Basierend auf dieser Thematik entstand die erste Studie, die den Aspekt des Tierwohls von Rosaflamingos (*Phoenicopterus roseus*) mit unterschiedlichem Flugstatus in deutschen Zoos wissenschaftlich evaluiert hat (Reese2020b). In dieser wurden Verhaltensbeobachtungen mit der Messung und Interpretation von Federcorticosteron verknüpft. Eine vergleichbare Studie wurde 2021 über Rosapelikane (*Pelecanus onocrotalus*) veröffentlicht (Haase et al. 2021).

## 2.2 Wissenschaftliche Untersuchung von Stress

### 2.2.1 Definition Stress

Eine exakte und beständige Definition des Begriffs „Stress“ ist in der Wissenschaft bis heute schwierig (Romero et al. 2009). Siegel (1980) beschreibt Stress als zwei neurohumorale Systeme, die durch innere oder äußere Reize aktiviert werden können: (1) das neurogene

System, das als unmittelbare Reaktion folgt und der schnellen Energielieferung dient; (2) das Zusammenspiel aus Hypothalamus, Hypophyse und Nebenniere (HPA-Achse), das für eine langsamere, aber langfristigere Mobilisierung der Abwehrkräfte verantwortlich ist. Beide Reaktionen sorgen für temporäre Abwehrmechanismen, die jedoch im Vogel einen unspezifischen Stresszustand auslösen und zu reduzierten Wachstumsraten und verminderter Widerstandsfähigkeit gegen Krankheiten führen können (Siegel 1980). Moberg und Mench (2000) definieren Stress als die biologische Antwort eines Individuums, welche durch auf die Homöostase wirkende Stressoren ausgelöst wird. Grundsätzlich ist festzuhalten, dass ein Stressor (auslösender Reiz) über die individuelle Wahrnehmung und Verarbeitung zu einer Reaktion (Stressantwort) führt (Levine 2005). Hierbei kann „guter Stress" (Eustress) mit einer für das Wohlergehen des Individuums nicht bedrohlichen Antwort von „schlechtem Stress" (Distress) unterschieden werden (Moberg und Mench 2000). Entsteht eine Überlastung des Systems, die sich bis zu pathologischen Auswirkungen entwickeln kann, spricht man von „chronischem Stress". Sypolsky et al. (2000) beschreiben hierzu die Stressantwort als Reaktion, um den Zustand der Homöostase aufrecht zu erhalten. Der Hauptmechanismus der Homöostase kann als „Stabilität durch Beständigkeit" beschrieben werden, in der Stress als Diskrepanz dazu fungiert (Levine 2005;Fink 2017). Andere Autoren unterstützen eine Erweiterung des Modells um die Allostase. Diese beschreibt eine „Stabilität durch Veränderung", bei der die Reaktion auf den auslösenden Reiz eine Anpassung zur Erhaltung der Homöostase ist (Sterling und Eyer 1988;Mcewen und Wingfield 2003). Die Veränderungen, die dazu nötig sind, werden als „Allostatic Load" bezeichnet. Wenn nun bei chronischem Stress die Summe der Anpassungen zu hoch ist oder zu lange andauert, wird vom „Allostatic Overload" gesprochen. Romero et al. (2009) erweiterten dieses Konzept in ihrem „Reactive Scope Model", das vier Reaktionen beinhaltet: vorhersehbare Homöostase, reaktive Homöostase, homöostatische Überlastung und homöostatisches Versagen.

### 2.2.2 Physiologie der Glucocorticoide bei der Stressantwort

Erfährt das Individuum einen auslösenden Reiz (Stressor), steigt im Blut die Konzentration von Glucocorticoiden (GC) (Sapolsky et al. 2000;Romero und Fairhurst 2016). Dieses Phänomen lässt sich auf eine Kaskade von Hormonausschüttungen zurückführen. Wirkt ein Stressor auf das Individuum, erfolgt anfangs eine erhöhte Freisetzung von Katecholaminen in den Blutkreislauf, die wiederum die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) stimulieren (Sapolsky et al. 2000;Vera et al. 2017). Siegel (1980) beschreibt den Ablauf der HPA-Achse, der mit einer durch den Stimulus ausgelösten Produktion von Corticotropin-

Releasing-Hormon (CRH) im Hypothalamus beginnt, welches die Hypophyse dazu anregt Adrenocorticotropin (ACTH) auszuschütten (siehe Abb.1).

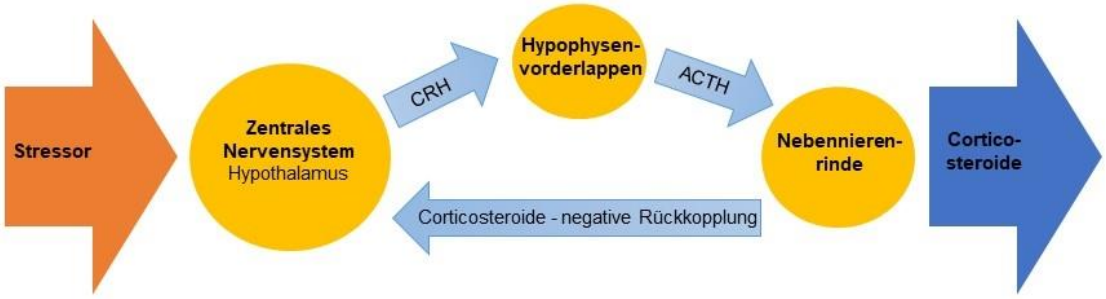


**Abbildung 1. Schematischer Überblick über die Hormonkaskade der HPA-Achse**
nach H.S. Siegel „Physiological Stress in Birds“ (1980).

ACTH gelangt über den Blutstrom zur Nebennierenrinde, wo es zu einer Proliferation der dort ansässigen Zellen kommt, die letztendlich Steroidhormone bilden und ausschütten (Siegel 1980). GC haben unterschiedliche Effekte auf die Physiologie des Vogels. Sie führen zu Symptomen, wie die Erhöhung der Herzfrequenz oder die Steigerung des Blutdrucks, und gleichzeitig zu einer geringeren Risikoaffinität des Tieres, wodurch das Überleben des Individuums gesichert wird (Sapolsky et al. 2000;Hau et al. 2010). Diese Reaktion wird als Kampf-oder-Flucht-Antwort (englisch: fight-or-flight response) bezeichnet (Sapolsky et al. 2000;Vera et al. 2017).

Allerdings sollten GC trotz ihrer wichtigen Rolle bei der Stressantwort nicht nur als „Stresshormone“ betrachtet werden, denn sie werden nicht nur in unterschiedlichen Konzentrationen als Reaktion auf interne oder externe Faktoren ausgeschüttet, sondern es besteht auch eine Grundkonzentration dieser Hormone im Blut (Cockrem 2013;Vera et al. 2017). Einflussfaktoren auf den Hormonhaushalt stellen beispielsweise das Alter, das Geschlecht oder der körperliche Zustand dar (Cockrem 2013). Die Ausschüttung an GC kann auch mit dem Klima oder der Jahreszeit variieren (Cockrem 2013). Die Auswirkungen von GC weisen in ihrer Funktion eine Heterogenität auf und können somit vorbereitend auf einen nachfolgenden Stressor wirken, stimulierend auf den Blutdruck und den katabole Stoffwechsel, unterdrückend auf das Reproduktionsverhalten oder permissiv auf den Verteidigungsmechanismus u.v.m. (Sapolsky et al. 2000). Bei Vögeln, Reptilien und Amphibien stellt CORT das wichtigste GC dar, wohingegen dieses bei Säugetieren und Fischen Cortisol ist (Cockrem 2013;Dickens und Romero 2013).

### 2.2.3 Corticosteron in Federn

#### *2.2.3.1 Bedeutung*

Die Messung von CORT in Federn (CORTf) findet immer mehr Anwendung in Studien zur Untersuchung von Stress bei Vögeln (Romero und Fairhurst 2016). Im Gegensatz zur Blutabnahme zur Untersuchung von CORT im Plasma gilt CORTf als weniger invasiv und ermöglicht einen Rückblick auf die Aktivität der HPA-Achse des Vogels während des Federwachstums (Kennedy et al. 2013;Romero und Fairhurst 2016). In der Zeit der Mauser zirkuliert Blut in den Federfollikeln, um das Wachstum zu ermöglichen. Hierbei diffundiert CORT in das Keratin der Feder und wird nach Austrocknung der Feder (Beendigung des Wachstums) darin eingelagert (Bortolotti et al. 2008). Die Studie von Bortolotti et al. (2008) beschreibt, dass ein erhöhter CORT-Spiegel im Plasma während des Federwachstums zu erhöhten CORTf-Werten führt. Dies wurde in anderen Arbeiten bestätigt (Lattin et al. 2011;Fairhurst et al. 2013;Jenni-Eiermann et al. 2015). Folglich korreliert die Menge des eingelagerten CORTf mit der Summe aus der Grundkonzentration an CORT im Plasma und durch Stimuli ausgeschüttetes CORT in der Phase des Federwachstums (Romero and Fairhurst 2016). Durch die Abbildung des Hormonhaushaltes über den relativ langen Zeitraum des Federwachstums (Tage bis Wochen) stellt die Messung von CORTf in einer trockenen Feder (somit ein abgeschlossenes Wachstum) einen klaren Vorteil für Untersuchungen von chronischem Stress dar. Diese Methode dient folglich der Betrachtung des gesamten Zeitraums des Federwachstums, wohingegen kurzfristige Belastungen oder Stresssituationen nicht beurteilt werden können (Bortolotti et al. 2008;Romero und Fairhurst 2016). Deshalb muss hier festgehalten werden, dass die Auswahl der Matrix (Plasma, Kot, Ei, Speichel oder Feder) zur CORT-Bestimmung von der wissenschaftlichen Frage abhängig ist. Die Verwendung der Feder als Matrix birgt den Vorteil, dass zur Lagerung der Proben keine weitere Bearbeitung vonnöten ist. Es genügt sie trocken und sauber bis zur Verarbeitung aufzubewahren (Bortolotti et al. 2009;Bortolotti 2010;Romero und Fairhurst 2016;Monclús et al. 2017;Monclús et al. 2018). CORTf ist über Jahre stabil in der Feder nachzuweisen und beständig gegen Hitzeeinfluss (Bortolotti et al. 2009). Daraus folgend ermöglicht die Methode der Messung von CORTf eine lange Lagerung, sodass beispielsweise Proben von seltenen Arten oder Wildtieren bei einer guten Gelegenheit gesammelt werden können, die anschließende Analyse jedoch zu einem späteren Zeitpunkt erfolgen kann.

#### *2.2.3.2 Messung*

Die Messung von CORTf wird mittels eines Enzyme-linked Immunosorbent Assay (ELISA) durchgeführt. Bei Verwendung dieses Nachweisverfahrens werden die Federproben

weiterverarbeitet. Hierzu wird sich exakt an das von Bortolotti et al. (2008) entwickelte und von Monclús et al. (2017) modifizierte Protokoll gehalten. Bei jeder Probenuntersuchung einer neuen Vogelart wird empfohlen zuvor eine Assay-Validierung vorzunehmen, um möglichst exakte Ergebnisse zu erhalten (Buchanan und Goldsmith 2004). Zu Beginn der Analyse werden Federn der zu untersuchenden Vogelart getestet, um herauszufinden wie viele Federn und welche minimale Gesamtlänge an Federn für die weitere Verarbeitung erforderlich sind und um die optimale Dauer in der Kugelmühle zur Herstellung eines feinen homogenen Pulvers zu ermitteln.

Nach der Festlegung dieser Eckdaten werden die Federn der einzelnen Proben nach gewünschter Gesamtlänge selektiert. Anschließend wird der Calamus jeder gerupften Feder abgeschnitten und die der geschnittenen Proben auf vollständiges Fehlen des Calamus überprüft (Buchanan und Goldsmith 2004;Bortolotti et al. 2008;Monclús et al. 2017). Darauffolgend werden die Proben auf 0,1 mg genau gewogen und danach in einer Kugelmühle (Retsch®, MM200 Typ mit zwei Kugeln und 25 Hz, Deutschland) für die bereits ermittelte Dauer zu einem Pulver mit einer Partikelgröße von ca. 10 µm zermahlen. Als wichtiger Kontrollschritt gilt es nun auch das Probenpulver zu wiegen, um sicherzugehen, dass es keinen großen Verlust an Material gibt. Das Federpulver jeder einzelnen Probe wird mit 1,5 ml Methanol in einem Vortex (Vortex Mixer S0200–230 V-EU; Labnet International, Edison, NJ, USA) bei Raumtemperatur für 30 Minuten vermischt. Das hergestellte Gemisch inkubiert bei 37°C für 18 Stunden in einem Schüttelinkubator (G24 Environmental Incubation Shaker, New Brunswick Scientific, Edison, NJ, USA). Im Folgenden wird die Probe für 15 Minuten bei 3500 x g (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany) zentrifugiert. Vom resultierenden Überstand wird 1 ml in ein Eppendorf® Reaktionsgefäß pipettiert. Anschließend wird diese Probe in einem Ofen (Heraeus Function Line T6®, Thermo Fisher Scientific, Waltham, MA, USA) bei 38°C getrocknet, woraufhin der bleibende Rückstand in 0,25 ml einer Pufferlösung, die BSA, NaCl, EDTA und Azid enthält und mit einem kommerziellen ELISA-Set (ELISA Neogen® Corporation, Ayr, UK) funktioniert, aufgelöst und danach in einem Vortex für eine Minute gemischt wird. Wird die Probe nicht direkt in den ELISA zur CORTf-Messung eingegeben, sollte sie bei -20°C bis zur Analyse gefroren werden. Nach Eingabe der Probe in den ELISA kommt es zu einer spezifischen Antigen-Antikörper-Bindung (Töpfer 2019). In einem weiteren Schritt wird eine enzymatische Farbreaktion auslöst, wodurch der Nachweis und die Quantifizierung von CORTf möglich ist (Töpfer 2019). Vor dem Durchlauf einer Probencharge wird die Tierart validiert und die Intra- und Inter- Assay-Variationskoeffizienten bestimmt, um die Genauigkeit und Wiederholbarkeit des Assays zu überprüfen. Folgt nun die Ablesung des ELISA, so sollte CORTf mit der Einheit pg pro mm Federlänge angegeben werden. Dies liegt begründet in der Annahme, dass die Federwachstumsrate in morphologisch

ähnlichen Federn eines Individuums und an der gleichen Probenentnahmestelle einheitlich ist (Bortolotti et al. 2009). Die Hormonexposition gilt somit als zeitabhängig, wodurch der CORTf-Wert durch Federlänge spezifiziert werden sollte (Bortolotti et al. 2008;Bortolotti et al. 2009;Rohwer et al. 2009;Bortolotti 2010;Romero und Fairhurst 2016).

*2.2.3.3 Einflussfaktoren*

Um CORTf korrekt beurteilen zu können, ist zum einen auf die Federprobenqualität an sich zu achten. Die zu untersuchende Feder sollte weder Blut noch Kot oder andere biologische Kontaminationen aufweisen, da diese ebenfalls CORT enthalten und die Probe somit verfälschen könnten (Kennedy et al. 2013). Ebenso kann die Federfarbe Einfluss auf den CORTf-Spiegel nehmen, weshalb in einer zu untersuchenden Gruppe nach Möglichkeit eine einheitliche Färbung gewählt werden sollte (Grindstaff et al. 2012;Kennedy et al. 2013;Lendvai et al. 2013;Fairhurst et al. 2014). Tallo-Parra et al. (2015) empfehlen in ihrer Studie, dass die Haarproben zur Cortisol-Messung bei Milchkühen homogen in Farbe und von einer einheitlichen Körperstelle gewählt werden sollten (Tallo-Parra et al. 2015). Überträgt man dies auf Vögel, so ist neben der einheitlichen Federfarbe, sowohl ein morphologisch identischer Federtyp, als auch die gleiche Entnahmestelle innerhalb und zwischen den Individuen zu wählen, um ein einheitliches Federwachstum aller Proben zu garantieren (Bortolotti et al. 2009).

Neben der Qualität der Probe beeinflussen externe Stressoren das Level an CORT in der Feder. Wirken Stressoren auf das Individuum ein, steigt der Gehalt an CORT im Plasma (Sapolsky et al. 2000). Zu den externen Einflussfaktoren werden auch der Lebensraum und dessen Qualität, sowie Umweltbedingungen, Jahreszeit und Fütterungsroutine gezählt (Astheimer et al. 1995;Marra und Holberton 1998;Romero et al. 1998;Bortolotti et al. 2008;Legagneux et al. 2013). Hau et al. (2010) beschreiben hierzu geringere CORT-Spiegel bei Vogelarten aus kälteren und trockeneren Gebieten.

Des Weiteren gilt als essenziell, möglichst genau die individuellen biologischen Daten der zu untersuchenden Vögel mit aufzunehmen, um den ökophysiologischen Grundzustand während der Periode der Hormonausschüttung zu erfassen (Romero und Fairhurst 2016). Faktoren wie das Alter, das Geschlecht, der körperliche Zustand, die Aktivität, die Mauser und weitere biometrische Merkmale beeinflussen die CORTf-Spiegel, daher ist die Erfassung dieser Daten für die Interpretation der Ergebnisse notwendig (Romero und Fairhurst 2016). Es werden unterschiedliche Reaktionen von Jungvögeln auf Stressfaktoren beschrieben, die zu einem variierenden Anstieg oder Abfall von CORT im Vergleich zu Erwachsenen führen (Dufty Jr und Belthoff 1997;Sims und Holberton 2000). Fairhurst et al. (2014) beschreiben einen niedrigeren CORT-Wert im Plasma bei adulten männlichen Rotkehlchen (*Acanthis flammea*) als bei

weiblichen oder juvenilen männlichen Individuen. Die geschlechtsspezifischen Unterschiede im CORT-Level werden jedoch oft nur in Zusammenhang mit der unterschiedlichen Färbung des Gefieders gesehen (Bortolotti et al. 2008;Grindstaff et al. 2012;Kennedy et al. 2013;Lendvai et al. 2013;Fairhurst et al. 2014). Da eine Korrelation des körperlichen Zustands eines Vogels mit CORT-Werten im Plasma festgestellt wurde, wird empfohlen die Gesundheit und Konstitution mit in die Interpretation von CORTf aufzunehmen (Angelier et al. 2009;Bortolotti et al. 2009;Harris et al. 2017).

Zusammenfassend sollten die zu untersuchenden Individuen während der Zeit des Federwachstums genau beobachtet werden, um externe Einflüsse auf den Vogel, Stressoren und individuelle biologische Daten in die Interpretation von CORTf mit einbeziehen zu können. Dies kann zur Schwierigkeit bei Wildvogelpopulationen werden. Wenn keine Beobachtungen zur Zeit der Mauser möglich sind, sollten möglichst genau biologische Daten der zu untersuchenden Vogelart und äußere Bedingungen, wie das Klima, in die Studie aufgenommen werden.

### 2.2.4 Die Probenentnahme der Federn

Zur Bestimmung von CORTf werden Federn von lebenden oder toten Vögeln entnommen oder sogar gemauserte verwendet (Bortolotti, Marchant et al. 2008, Fairhurst, Frey et al. 2011, Lattin, Reed et al. 2011, Kennedy, Lattin et al. 2013). Der bisherige Standard der Federentnahme zur CORTf-Bestimmung bei lebenden Vögeln ist das Federrupfen. Die Feder sollte trocken (Wachstum abgeschlossen) und sauber sein. Ist die Feder verschmutzt (mit Blut oder Kot) oder enthält sie noch Blut im Calamus, kann der CORTf-Spiegel nicht nur auf die Zeit des Federwachstums zurückgeführt werden (Kennedy, Lattin et al. 2013). Zur Vermeidung weiterer Fehlinterpretationen, sollten die Probennehmer Handschuhe tragen, damit die Federn nicht in Kontakt mit Schweiß kommen, da auch dieser die Werte verfälschen könnte.

Um eine möglichst genaue Standardisierung in den Studien zu erhalten, wird nicht nur auf eine einheitliche Federprobenqualität (z.B. Farbe, Morphologie) geachtet, sondern auch die gleiche Lokalisation bei der Probenentnahme gewählt: zwischen den Schulterblättern (Voit et al. 2020;Voit et al. 2021). Dieser Bereich wurde schon in vorherigen Projekten bei Rosaflamingos (Reese et al. 2020b), Rosapelikanen (Haase et al. 2021), Rotmilanen (Monclús et al. 2018) und Masthähnchen (Carbajal et al. 2014) validiert, da dort bei Entnahme von Federn die Flugfähigkeit des Vogels nicht beeinträchtigt wird und die Stelle leicht zugänglich ist.

### 2.2.5 Die Problematik der Federprobenentnahmemethode

Um Hormone in Federn zu bestimmen, war in vielen Studien der bisherige Entnahmestandard die Federn zu rupfen. Diese Probenentnahmemethode gilt bei lebendigen Vögeln als traumatisierend und wird als schmerzhaft interpretiert (Gentle 1992;Gentle und Hunter 1991;Gentle et al. 1990;Malik und Valentine 2018). Gentle (1992) stellt das Federrupfen als schmerzhaften Eingriff dar, der zu Änderungen der Physiologie und des Verhaltens führt. Durch die nozizeptive Stimulation folgt ein Anstieg der Herzfrequenz und des Blutdrucks (Gentle 1992). Ebenso können eine hohe Amplitude im Elektroenzephalogramm und schmerzbezogene Verhaltensmuster beobachtet werden (Gentle 1992).

Aufgrund dieser Begebenheiten ist in Deutschland, wie auch in anderen europäischen Ländern, die Beantragung eines Tierversuchs bei der zuständigen Behörde vor der Durchführung der Probenentnahme notwendig. Dies stellt die Wissenschaftler vor mehrere Herausforderungen. Zum einen ist die Grundlage der Entwicklung einer Studie zur Untersuchung von CORTf meist die Erforschung und Beurteilung des Wohlbefindens der beprobten Vögel. Daher ist wünschenswert, Schmerzen durch die Technik des Federrupfens selbst in Zukunft zu vermindern oder zu vermeiden. Nur so können die Wissenschaftler ihrer gesetzlichen Verpflichtung (EU Richtlinie 2010/63/EU, TierSchG und TierSchVersV) nachkommen und im Sinne des 3-R-Prinzips handeln, um Tierversuche zu verbessern und deren Zahl zu reduzieren (Russell et al. 1959;Directive 2010/63/EU).

## 2.3 Statistische Grundlagen

In den zwei Veröffentlichungen dieser Arbeit wurde eine Mindestprobenmenge von 45 Tieren pro Vogelart aufgrund einer biometrischen Planung im Vorfeld festgelegt. Diese Stichprobengröße führte zu einem Äquivalenztest der Mittelwerte mit einer Aussagekraft von 90% und einem Signifikanzniveau von 5,0%, wohingegen der wahre Unterschied der Mittelwerte bei 0,0% lag (Voit et al. 2020;Voit et al. 2021).

Bei den ausgewählten Vögeln der beiden Studien wurden die Federproben bei jedem Individuum auf zwei verschiedene Arten entnommen: Zum einen wurden sie nach dem bisherigen Standard gerupft, zum anderen mit der zu validierenden, weniger invasive Technik hautnah abgeschnitten. Somit ist jedes Tier für diesen Vergleich seine eigene Kontrolle.

Die Bestimmung von CORTf erfolgte mittels ELISA. Es sollte vor der Analyse und folgender Interpretation die Genauigkeit des Tests mittels des Intra- und Inter- Assay-Variationskoeffizienten überprüft werden. Dazu wurde ein Pool aus zehn verschiedenen

Proben von zehn verschiedenen Individuen erstellt. Dieser lief dreimal durch jeden Assay, wohingegen die eigentlichen Proben zur CORTf-Messung den ELISA einmalig durchliefen.

Werden zwei Techniken zur Probenentnahme verglichen, erfolgt im ersten Schritt die genaue Auswertung der CORTf-Level. Hierbei wurden die Maximal-, Minimal- und Durchschnittskonzentrationen an CORTf in pg/mm verglichen, ebenso wie die Durchschnittswerte von Gesamtlänge und Gewicht. So ließen sich Trends zwischen den Methoden und den Geschlechtern erkennen. Um die Vergleichbarkeit zwischen den zwei Techniken zu ermöglichen, wurden die beiden Methoden innerhalb jedes einzelnen Individuums verglichen. Dafür wurden mehrere statistische Untersuchungen angewendet.

### 2.3.1 Konkordanz-Korrelationskoeffizient

In der Abbildung des Konkordanz-Korrelationskoeffizienten stellt ein Punkt ein Individuum mit seinem jeweils gerupften und geschnittenen CORTf-Wert dar. Daraus ergibt sich eine Grafik, die den Grad der Abhängigkeit der zu untersuchenden Werte von der 45°-Linie durch den Ursprung darstellt (Lawrence und Lin 1989). Diese Linie entspricht der perfekten Übereinstimmung von zwei Methoden (Lawrence und Lin 1989). Daraus folgend misst der Konkordanz-Korrelationskoeffizient die Präzision und Genauigkeit der Prüfmethode.

In dieser Grafik wird der Pearsons-Korrelationskoeffizient mitverarbeitet. Dieser ermittelt, ob eine lineare Abhängigkeit zwischen zwei Variablen vorhanden ist. Beträgt der Wert 0, liegt keine lineare Abhängigkeit vor und die Linie verläuft grafisch horizontal.

### 2.3.2 Bland-Altmann-Plot

Bei dieser statistischen Untersuchung werden die Differenzen zwischen den CORTf-Werten beider Entnahmetechniken eines Individuums in Relation zu den jeweiligen Mittelwerten gesetzt und grafisch dargestellt. Aus der Mittellinie, die die mittlere Differenz darstellt, des 95%-Konfidenzintervalls und der errechneten Werte ergibt sich ein anschauliches Bild. Es dient der Beurteilung von Tendenzen (aufsteigend oder absteigend), der Übereinstimmung beider Methoden und der Identifizierung von Unterschieden und Ausreißern (Bland und Altman 1986;Bland und Altman 1999).

### 2.3.3 Mann-Whitney U Test

Um die Möglichkeit eines Geschlechtsunterschieds in CORTf-Spiegeln zu ermitteln, wird der Mann-Whitney U Test angewendet. Hierbei wird das jeweilige Geschlecht mit CORTf in

Relation gesetzt. In der veröffentlichten Studie dieser Arbeit wurde der Test mit der zweiseitigen t-Approximation gewählt, da sich die Gesamtheit der Werte als nicht-normalverteilt darstellten (Mcknight und Najab 2010;Voit et al. 2021). Die Ergebnisse der Untersuchung werden in einem Box-Plot-Diagramm verbildlicht und verglichen.

## 2.4 Die Versuchsgruppen

Bei der Validierung einer neuen Probenentnahmetechnik sollte die Versuchsgruppe in ihren biologischen Daten und Lebensbedingungen hinsichtlich der Aspekte, die mögliche Veränderungen der CORT-Spiegel induzieren können, ähnlich sein (Voit et al. 2020). So kann davon ausgegangen werden, dass die gesamte Gruppe in der Regel die gleichen Stressoren innerhalb der Zeit des Federwachstums erfährt und somit die Einflussfaktoren entsprechend der jeweiligen artspezifischen ethologischen Bedürfnisse gleichbleiben. Es ist zu erwarten, dass die beeinflussenden Faktoren auf den CORT-Spiegel dadurch so weit wie möglich standardisiert werden, sodass im Rückschluss die Unterschiede innerhalb der Spiegel verstanden werden können (Voit et al. 2020). Der Vergleich der beiden Probenentnahmemethoden ist somit besser durchführbar, da die Variation innerhalb und zwischen den Gruppen so gering wie möglich gehalten wird. Nichtsdestotrotz können individuelle Unterschiede in der Grundkonzentration an CORT auftreten, ebenso wie spezifische individuelle, stressbedingte Spitzenwerte. Hinzuzufügen ist, dass die zweckmäßige und zufällige Stichprobenstrategie bei der Auswahl an Individuen wichtig für ein repräsentatives Ergebnis ist.

### 2.4.1 Studie 1: Geflügel aus einer konventionellen Freilandhaltung

Im ersten Projekt wurden Hausgänse (*Anser anser domseticus*) und Mularden-Enten (*Anas sterilis* bzw. *Cairina moschata domestica* x *Anas platyrhynchos domesticus*), ein Hybrid aus der Moschusente (*Cairina moschata domestica*) und der Pekingente (*Anas platyrhynchos domesticus*), zur Untersuchung ausgewählt (Voit et al. 2020). Die Auswahl wurde vor dem Hintergrund getroffen, dass diese beiden Vogelarten der Ordnung der Gänsevögel (Anseriformes) angehören. Diese stehen auf verschiedenen Auflistungen von Vogelarten, bei denen eine genauere ethologische und physiologische Untersuchung der Relevanz von Flugfähigkeit zur Beurteilung des Wohlbefindens empfohlen wird (Dollinger et al. 2013;TVT 2015).

Des Weiteren brachte die Haltung (konventionelle Freilandhaltung zur Fleischproduktion) den Vorteil, dass eine große Anzahl an Tieren zur Beprobung vorhanden war und die beiden

Versuchsgruppen zur Zeit der Probenentnahme im selben Alter waren. Da beide Vogelarten zusammen im Freilandgehege gehalten wurden, ist davon auszugehen, dass die Tiere im Durchschnitt, mit Ausnahme von individuellen Unterschieden und spezifisch erfahrenen Stressoren, die gleichen externen Einflussfaktoren in der Zeit des Federwachstums erfahren hatten. Sowohl die Gänse als auch die Enten besaßen ein weißes Federkleid, wodurch die Standardisierung der Federproben in der Farbe gegeben war. Der Aspekt eines möglichen Unterschieds an CORT-Werten von Geschlechtern wurde in dieser Studie nicht berücksichtigt.

Die Probenentnahme wurde nach der Schlachtung der Tiere zur Lebensmittelgewinnung durchgeführt. Dies ermöglichte ein Federrupfen ohne vorherige Beantragung eines Tierversuchs bei der zuständigen Behörde.

### 2.4.2 Studie 2: Vogelarten aus der Wildbahn

Die Tatsache, dass zur Interpretation von CORTf die genauen biologischen Daten, die Mauserzeit und das Verhalten zur Zeit des Federwachstums essenziell sind, stellt die Wissenschaftler bei Wildvögeln vor eine Herausforderung. Deshalb ist es besonders wichtig, alle Daten und Gewohnheiten der Vogelart dieser Population in die Untersuchung zu integrieren.

In der zweiten Studie wurden Individuen der beiden Wildvogelarten Rosaflamingo (*Phoenicopterus roseus*) und Stockente (*Anas platyrhynchos*) beprobt (Voit et al. 2021). Auch bei diesen beiden Ordnungen, Anseriformes und Phoenopteriformes, fordern diverse Veröffentlichungen eine weiterführende wissenschaftliche Untersuchungen des Wohlbefindens von in Menschenobhut gehaltenen Vögeln dieser Art mit unterschiedlichem Flugstatus (Dollinger et al. 2013;TVT 2015).

Die Gruppe der Flamingos wurde im Zuge einer Beringungsaktion in Marismas del Odiél, Andalusien (Spanien), beprobt. Die dort ansässige Population wird jährlich kontrolliert und überwacht (Rendón-Martos et al. 2009). Das Fangen und Beproben der Flamingos lief innerhalb eines staatlich geförderten Projekts ab und ist von den dort ansässigen Behörden genehmigt (Junta de Andalucía 2019;Fundación Unicaja 2019). Die untersuchten Vögel waren juvenil, alle noch im grauen Federkleid und wurden auf ein Alter von vier bis zehn Wochen geschätzt.

Die zu untersuchenden Stockenten sind in der Region „Nürnberger Land“, Bayern (Deutschland), beprobt worden. Durch die jagdliche Beschießung war es möglich die Federn nach dem Tod und vor der weiteren Verarbeitung zu entnehmen. Die beprobten Enten waren adult. Bei der Beprobung wurde darauf geachtet, dass nur braune Federn entnommen wurden.

Ein möglicher Geschlechtsunterschied in den CORTf-Werten wurde in dieser Studie mitberücksichtigt. Die Stockenten zeigten zu dieser Jahreszeit einen sichtbaren Geschlechtsdimorphismus. Wohingegen bei den juvenilen Flamingos eine DNA-Analyse der Federn vorgenommen wurde.

### 2.5 Ziel der Studien

Durch die Validierung von „hautnah Schneiden“ als Federentnahmetechnik zur CORTf-Bestimmung handeln die Wissenschaftler zukünftig bei Studien, die diese Methodik enthalten, im Sinne der Vorgaben im nationalen Tierschutzrecht und des EU Rechts und damit der Umsetzung des 3-R-Prinzips (Tierschutzgesetz 1972;Russell et al. 1959;Directive 2010/63/EU;TierSchVersV 2013). Die Verfeinerung (Refinement) der Methode bedeutet ein Handeln im Sinne des Tierwohls, da so dem zu untersuchenden Vogel die Schmerzen des Federrupfens erspart werden (Gentle 1992;Gentle und Hunter 1991). Darüber hinaus werden Studien zum Wohlbefinden und der Stressphysiologie von Vögeln mittels Federschneiden ohne die Beantragung eines Tierversuchs möglich, weil diese Federprobenentnahmetechnik nicht mit Schmerzen, Schäden und Leiden für das Tier einhergeht, so wird der Verringerung von Tierversuchen Rechnung getragen.

Im Rückkehrschluss beinhaltet dies auch einen Vorteil für die Planung und Umsetzung von Studien. Somit können Vogelgruppen in zoologischen Gärten an einem unkomplizierteren und weniger invasiven Monitoring des Wohlbefindens teilnehmen. Dies bedeutet, dass bei Aktionen wie der Beringung oder der Einwinterung die Federn ohne größeren Aufwand und zusätzlichen Stress für den Vogel geschnitten werden können. Zusätzlich stellt der Wegfall eines Tierversuchsantrages eine Erleichterung der Untersuchung von Wildtieren dar. Es besteht nun die Möglichkeit mit Auffangstationen für Wildvögel zusammenzuarbeiten. Die Stellung eines Tierversuchsantrages würde sich in diesem Fall als eine größere Herausforderung darstellen, da im Voraus nicht zu sagen ist, an welchem Ort wie viele Tiere beprobt werden. Durch eine gesteigerte Beprobung von Wildvögeln wird eine größere Datengrundlage an CORTf und damit eine Kontrollgruppe zu in Menschenobhut lebenden Vögeln möglich.

Insgesamt dient diese Arbeit zusätzlich der Sammlung von CORTf-Daten von verschiedenen Vogelgruppen, um eine gute Interpretation von Messungen in zukünftigen Studien zu ermöglichen.

## 3 Validation of an Alternative Feather Sampling Method to Measure Corticosterone

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Abstract:

The most common feather sampling method for feather corticosterone measurement is by plucking the feathers from the bird's skin. This procedure performed on living, restrained birds is qualified as an animal experiment according to German/European legislation, which has to be applied for from the competent authorities. The Directive 2010/63/EU requires the full implementation of the 3-R Principle of Russel and Burch in animal experiments, which means not only to replace the use of animals, but also to reduce the number of animals used and to refine procedures whenever possible. In response to this issue, the aim of this study was to

validate an alternative, less invasive sampling method by cutting feathers close to the skin in comparison to the gold standard of plucking them. For this proof-of-principle study, a conventional poultry husbandry with trial groups of geese (*Anser anser domesticus*) and ducks (*Anas sterilis*) was selected. All birds were kept under the same living conditions to standardize the influencing factors regarding husbandry, and thus, their stress levels. Feather samples were collected between the shoulders from 46 geese and 51 ducks, both by cutting as well as by plucking, directly after slaughter for meat production. Feather corticosterone levels were measured with Enzyme-Linked Immunosorbent Assay (ELISA). Results were compared using Bland–Altman plots and concordance correlation coefficients (CCC). It could be seen that concordance between corticosterone levels in cut and plucked feathers was rather poor: 0.38 for *Anser*, and 0.57 for *Anas*. However, comparing the mean corticosterone values in pg/mm of each species with their respective standard deviations, the differences between the methods were negligible. As the results showed that the differences between the individuals were markedly greater than the differences between the methods, the determination of corticosterone levels in cut feathers is valid compared to using plucked feathers. The validation tests of ELISA showed only acceptable repeatability and reliability. Hence, the results should be verified in further studies. In conclusion, it is recommended for future research to use cut instead of plucked feathers for corticosterone measurement.

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*Article*

# Validation of an Alternative Feather Sampling Method to Measure Corticosterone

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**Simple Summary:** Research projects on birds' welfare or their stress physiology are often complemented by measurements of corticosterone level in feathers. Until now, the common standard for this method is to collect the feathers by plucking, a procedure which on living birds is presumed to be painful and to cause stress. Therefore, in most European countries an animal experiment application is required. The aim of this study was to validate an alternative, possibly less stressful sampling method: cutting the feathers close to the skin. The examined species were geese and ducks from a conventional poultry husbandry. There was no relevant difference between the two methods assessed according to statistical analysis. In conclusion, it is reasonable to assume that feather cutting could be established as an alternative sampling method for measuring corticosterone. Nevertheless, we recommend further research on other species to confirm these results.

**Abstract:** The most common feather sampling method for feather corticosterone measurement is by plucking the feathers from the bird's skin. This procedure performed on living, restrained birds is qualified as an animal experiment according to German/European legislation, which has to be applied for from the competent authorities. The Directive 2010/63/EU requires the full implementation of the 3-R Principle of Russel and Burch in animal experiments, which means not only to replace the use of animals, but also to reduce the number of animals used and to refine procedures whenever possible. In response to this issue, the aim of this study was to validate an alternative, less invasive sampling method by cutting feathers close to the skin in comparison to the gold standard of plucking them. For this proof-of-principle study, a conventional poultry husbandry with trial groups of geese (*Anser anser domesticus*) and ducks (*Anas sterilis*) was selected. All birds were kept under the same living conditions to standardize the influencing factors regarding husbandry, and thus, their stress levels. Feather samples were collected between the shoulders from 46 geese and 51 ducks, both by cutting as well as by plucking, directly after slaughter for meat production. Feather corticosterone levels were measured with Enzyme-Linked Immunosorbent Assay (ELISA). Results were compared using Bland–Altman plots and concordance correlation coefficients (CCC). It could be seen that concordance between corticosterone levels in cut and plucked feathers was rather poor: 0.38 for *Anser*, and 0.57 for *Anas*. However, comparing the mean corticosterone values in pg/mm of each species with their respective standard deviations, the differences between the methods were negligible. As the

results showed that the differences between the individuals were markedly greater than the differences between the methods, the determination of corticosterone levels in cut feathers is valid compared to using plucked feathers. The validation tests of ELISA showed only acceptable repeatability and reliability. Hence, the results should be verified in further studies. In conclusion, it is recommended for future research to use cut instead of plucked feathers for corticosterone measurement.

**Keywords:** feather corticosterone; comparative study; plucked feathers; cut feathers; Domestic Goose; Mulard Duck; animal welfare

## 1. Introduction

The technique of measuring corticosterone in feathers enables a long-term reconstruction of hormone exposure during feather growth. Feather growth has a timeframe ranging from days to weeks, as it depends on the species, the type of feather and the life history stage of the individual [1]. The steroid hormone corticosterone is released from the adrenal gland in baseline concentrations and in response to perceived or actual stress stimuli [1,2]. An acute rise in corticosterone concentration protects the bird by promoting those behaviors that reduce risk and is associated with a higher annual adult survival rate [3]. However, chronic high concentrations of corticosterone are detrimental to birds' welfare and can lead to reproductive failure, low growth rate or neuron death [2,4–6]. In consequence, corticosterone serves as a very useful physiological indicator of the impact of stress stimuli for scientific investigations [7].

Plasma corticosterone is subject to diurnal variations and increases in stressful situations. Therefore, repeated blood samples are necessary to show an expressive hormone course. On one hand, it may result in underestimating hormone exposure because of hemodilution, already present after 15 min during the second bleeding [8]; on the other hand, it may cause a higher stress level due to repeated capture of the birds. Whereas measuring feather corticosterone is, especially in cut feathers, a non-invasive technique that gives the researcher the opportunity to gain a retrospective and long-term view of a bird's corticosterone exposure. However, the method is limited in reflecting just the time of feather growth, whereby short-term stress cannot be estimated [1,9]. In conclusion, due to this fact, the measured values mirror only the sum of the hypothalamic-pituitary-adrenal axis (HPA axis) activity including the stress incidents that the bird experienced in this period.

Next to these two methods of corticosterone measurement, many reports exist about other matrixes that may be used as bases for stress-level studies. The examination of feces plays an essential role [10,11]. For correct interpretation of the corticosterone metabolites in birds' droppings, the gut passage time needs to be known to draw conclusions about the timeframe of exposure. For example, Kotschral et al. describe a short gut passage time of two to three hours in domestic geese [12]. Eggs can also be used for hormone-level measurement to show stress in egg-laying birds during egg production [13,14]. Saliva has proved to be unreliable as an ideal medium for corticosterone measurements in birds [15]. In summary, it depends on the study objective which question is asked and thus, which medium is most suitable for the measurements.

Consequently, the amount of corticosterone embedded is correlated with the baseline level of corticosterone and stressor-related levels experienced by the bird during feather growth [1]. During molt, the feather follicles are supplied with blood to enable feather growth. As soon as the growth is accomplished the feather dries out, but the corticosterone is stored in the keratin. Therefore, the measured amount of corticosterone reflects the exposure to the hormone when blood circulates in the feather [1].

In recent studies, hormones have mainly been measured in plucked feathers. Using this sampling method presents a problem: the removal of feathers from living birds is traumatic and is thus interpreted as painful [16–19]. Gentle's book 'Pain in Birds', lists feather removal as a painful intervention [18].

It describes behavioral and physiological changes to nociceptive stimulations: increasing heart rate and blood pressure, a high amplitude in the electroencephalogram and behavioral patterns related to pain [18]. Therefore, an animal experiment application is currently required in Germany, which partly implies a long delay between application and permission. However, most importantly, the welfare of the animal should be paramount. In consideration of Directive 2010/63/EU, the number of animal experiments should be reduced and the 3-R Principle should be followed [20,21]. In conclusion, the cutting of feathers would be an alternative and less-invasive method.

Not only do external stressors affect the hormone level but individual biological data are essential to interpret the corticosterone level. Romero and Fairhust elucidate that "corticosterone data is greatly improved if the researcher can characterize the basic ecophysiological state of the bird as close as possible to the period of hormone deposition" in the feather [1]. Therefore, it is important to include factors such as age, sex, body condition, activity, molt and other biometrics to understand and eventually standardize variation in feather corticosterone in the study [1]. Several projects describe different reactions of young birds to stressors resulting in varied increasing or decreasing blood corticosterone in comparison to adults [22,23]. In addition, sex may also be an influencing factor. Common Redpolls (*Acanthis flammea*) were tested and showed lower plasma levels in adult males than in females or young males [24]. Additionally, in Snow Petrels (*Pagodroma nivea*), stress-related plasma corticosterone correlated negatively with body condition in females, but not in males [25]. Thus, because corticosterone could be affected by body condition, the health of the animal should not be disregarded [26,27]. Furthermore, the external factors that should be mentioned as influencing the corticosterone level are the habitat and its quality, as well as environmental conditions, season, and feeding routine [9,28–31]. Hau et al. describe a variation of stress-induced corticosterone that is related not only to different body masses and survival rates, but also to lower levels commonly seen in species of colder and drier environments [3]. When conducting experiments, the trial group should be similar in its biological data and living conditions concerning aspects which induce possible corticosterone level changes. Thereby, the whole group experiences the same stressors and, thus, maintains the influencing factors equal according to their species-specific ethological needs. Hence, standardizing the influencing factors on corticosterone levels, as far as possible, and understanding the differences within the levels will be expected. Consequently, the comparison of two methods is carried out in a better way because the variation within and between the groups is kept as low as possible. Of course, individual differences in the baseline of corticosterone can still occur, as can specific stress-induced peaks.

Geese and ducks were selected for this study because the order Anseriformes is mentioned on different lists of bird species for which ethological and physiological research on the relevance of flying in assessing wellbeing is recommended [32,33]. Furthermore, conventional poultry farming was chosen because the birds are slaughtered for food purposes, there was a sufficiently large number of them and—most importantly—they were of the same age and experienced the same living conditions.

Nowadays, aspiring knowledge about different bird husbandries and their respective animal welfare concerns is a clear objective in zoological institutions. New research projects have been conducted to examine the physiological and behavioral effects of deflighting procedures on zoo birds to assess the birds' wellbeing. One possibility is the measurement of corticosterone levels in feathers. The practice of deflighting birds in zoological institutions is regularly performed all around the world [34,35] but has become an increasingly pertinent issue due to legislative changes and increasing public awareness of animal welfare. Reese et al. give an overview of the relevance of this technique in combination with potential ethological and welfare concerns and furthermore pointing out the legal regulations in different countries [36]. As an example, in Germany, Article 6 of the Animal Welfare Act states that the total or partial amputation of parts of the body or the total or partial removal or destruction of organs or tissues of a vertebrate animal is prohibited [37]. In May 1998, the law was revised omitting the section on exceptions to the ban on amputation, "unless it is necessary for husbandries". The practices of deflighting zoo birds are increasingly criticized all over Europe, even though each single country has a different legal interpretation. Many authors

describe deflighting as a violation of animal welfare and the right to physical integrity should be legally protected [36,38–41]. However, from a different point of view, keeping birds deflighted could be, in some cases and in some species that are less dependent on or even independent of the ability of flight, considered necessary to protect them from escaping and to keep them in a better way according to their own biology [32,42]. The EU Zoos Directive 1999/22/EC defines the duties and responsibilities of zoological institutions as conservation, education and research. In conclusion, these institutions have, on the one hand, to take into account the individual needs of the respective species and, on the other, to prevent them from escaping [43]. Next to closed enclosures such as aviaries and ecosystem halls, deflighting birds for outdoor enclosures is often a way to enable birds to be kept in such institutions; therefore, a balance between the option to keep them in aviaries or in open-space enclosures and the welfare of the animals must be found [32,35]. Zoos are part of many European and international conservation breeding programs for which unified regulations concerning flightless birds and therefore science-based animal welfare assessments should be encouraged [32,36,43]. Associations such as the World Association of Zoos and Aquariums (WAZA) or, more regionally, the European Association of Zoos and Aquaria (EAZA), aim to introduce an international ethical code for zoos. Their guidelines provide a foundation for appropriate husbandries that should allow the animal to express its principal natural behaviors [44,45]. In addition, nationwide associations such as the German Veterinary Association for Animal Protection (Tierärztliche Vereinigung für Tierschutz—TVT) state the need for more ethological and physiological research into different husbandries [33].

In consequence, several ongoing research projects are evaluating different deflighting and husbandry practices of captive birds with respect to animal welfare concerns. In order to assess the wellbeing of these birds, the studies evaluate behavioral observations in combination with corticosterone measurements from feathers. This project aims to provide a basis for future studies on zoo birds and their relatives living in the wild.

The objective of this study was to investigate whether feather plucking could be replaced by feather cutting. In conclusion, after approval of the competent authority an animal experiment application is not necessary for cutting the feathers of living birds. Next to reducing the pain for the individual, another issue encourages the aim of this project. Following the protocol for the measurement of corticosterone in feathers, the calamus needs to be cut off as a first step of the protocol, even before measuring the total feather length of the sample. Regarding those parts of the feathers that are used for the analyses, they are in fact similar if either cut or plucked. Hence, the hypothesis is that there is no relevant difference in the corticosterone level between plucked and cut feathers and therefore cutting is a suitable alternative sampling method.

## 2. Materials and Methods

### *2.1. Subjects*

For this project, two species of waterfowl of the order Anseriformes were chosen: The Domestic Goose (*Anser anser domesticus*) and the Mulard Duck (*Anas sterilis*)—a hybrid of the Muscovy Duck (*Cairina moschata domestica*) and the Pekin Duck (*Anas platyrhynchos domesticus*).

The feather sampling took place directly after slaughter. As a sampling site, the region between the shoulders was chosen. These geese and ducks had been kept in an open-air enclosure. Just catching them for the sampling would have been very stressful for the animals as would holding them tightly for feather removal. Despite this, the plucking of feathers itself is likely to be painful to the birds [17–19]. Removing the feathers directly after slaughter allowed for an organized allocation of the individuals to the two sampling methods and for plucking them without an animal experiment application, according to the 3-R Principle [20,21] and, as a positive effect, a shorter legal method without the processing time of an application.

To create a conclusive study for the comparison of plucked and cut feathers, there is the need to use a trial group with preferably similar characteristics [46–48]. In the chosen groups of ducks and geese,

the individuals of each species were of the same age. Both chosen species did not show prominent sexual dimorphism, whereby this factor was not considered in this research project. However, sex disparity of corticosterone levels is often only put in context with different gender plumage hue [9,24,49–51]. Nevertheless, control of sex should be taken into account in further studies (if the sex is not obvious, e.g., via DNA analysis in feathers). The plumage of both species is mainly white in color.

### *2.2. Husbandry*

According to the guidance of the "Standing Committee of the European Convention for the Protection of Animals Kept for Farming Purposes", the husbandry of geese and ducks was evaluated as follows [47]:

All geese and duck chicks were bought from a wholesaler settled in northern Germany in mid-July 2018. The animals were transported in a conventional manner in accordance with transport legislation. At purchase, the age of the ducks was three weeks and the geese four to five weeks. They were transferred directly to a pasture measuring 5500 $m^2$ with an extension area of 4500 $m^2$, resulting in minimum 0.05 $m^2$/individual of a total of 130 geese and 120 ducks. The pasture was enclosed with an electric fence. The ground consisted mainly of grassland; the rest was earthy. This gave the chicks the opportunity to search for plants and small invertebrates. The area was big enough to guarantee that the geese and ducks had enough space to flap their wings, to perform their eating and drinking habits and to allow a plumage plastering movement.

The enclosures were provided with several shelters, such as old tractor-trailers, with enough space for the whole group, which served as sun protection, as well as protection from other weather conditions, environmental hazards and birds of prey.

Several water troughs and many smaller water buckets were distributed in the pasture. The water in the containers was deep enough to cover the whole head with water, so every goose and duck had the opportunity to reach water to drink or to clean their plumage at all times. As an enrichment to the measure, there was the possibility to expand the ground to a small stream. The troughs were cleaned regularly once a day and moved around so that the ground did not get too muddy.

In the first months, the geese were separated from the ducks because of different feeding routines and composition between the two species (see Table 1). The geese were fed mainly with oats. In contrast, the ducks received wheat. In the first month, both received special breeding feed (Gallugold® Enten-/Gänsekorn). Both always had free access to water and the pasture. They were held together in the enclosure in the last eight weeks before slaughter, resulting in a total fattening time of 4 to 5 months.

**Table 1.** Feeding schedule: overview of the different phases of the rearing feeding of the two species Domestic Goose (*Anser anser domesticus*) and Mulard Duck (*Anas sterilis*).

| Fattening Period | Geese | Ducks | Additional Information |
|---|---|---|---|
| 1. About two weeks | Crushed oats, pressed feed (Gallugold® Enten-/Gänsekorn), fattening feed for geese (Gallugold® Enten-/Gänsekorn) | Crushed wheat, pressed feed (Gallugold® Enten-/Gänsekorn) | Periods 1 to 4: Water and pasture ad libitum |
| 2. About two weeks | Oats, pressed feed (Gallugold® Enten-/Gänsekorn) | Wheat, pressed feed (Gallugold® Enten-/Gänsekorn) | Periods 1 to 3: Geese and ducks separated |
| 3. One to two months | Oats | Wheat | |
| 4. Final weeks before slaughter | Oats, wheat | Oats, wheat | Period 4: Geese and ducks together |

Prophylactic deworming was conducted twice in the time of rearing with levamisol (Concurat®-L 10%, Bayer Vital GmbH, Leverkusen, Germany; 40 mg levamisol per kg body weight dissolved in the drinking water).

There was a quarantine area available where sick individuals could be separated from the healthy flock. To assess the health of the whole poultry group, it was necessary to know the mortality rate (geese 2.3%, ducks 2.6%). In conclusion, the poultry keeper checked on the geese and ducks at a minimum twice a day to evaluate their health and body condition and to ensure the supply of water and food. Through the regular controls, the animals became quite accustomed to contact with humans.

To calculate the stage of feather growth for this research project, it was important that the molting of cover feathers was observed around six weeks before slaughter and at an estimated age of 4 to 5 months. The slaughter and feather sampling were performed at least six weeks after molting in order to guarantee sufficient feather growth. Slaughter took place on 18 November and December 2018. Catching before transportation took place directly before the slaughter date. Food and water were withheld only during transportation. Special poultry transport boxes were used for the journey. Two geese or four ducks were transported in each box. The distance from the enclosure to the slaughterhouse was around 9 km. During the slaughtering process, the birds were first placed in funnels and then stunned by a blow to the head. Afterwards, they were bled through a throat cut.

### *2.3. Sampling Protocol*

Feather sampling took place directly after slaughter. The initially sampled 46 geese and 51 ducks were selected randomly from the total of 130 geese and 120 ducks. Three to five feathers with a minimum collective length of 20 cm per sample were both plucked and cut from every single individual between the shoulders. Three or four feathers from each goose and four to five feathers from each duck were collected for each sampling method: plucked and cut. While plucking, the researcher had to be careful that the feathers did not contain any blood and that the quills were dry. The cover feathers needed to be clipped as close as possible to the skin. In addition, to standardize the sampling site between the birds, every feather sample was cut and plucked from the interscapular region.

To protect the feathers from contamination the person in charge of sampling was wearing gloves. The researcher also tried to ensure that no other biological fouling, such as feces or blood, was present. The feather samples were stored in paper envelopes at room temperature [1].

### *2.4. Analysis of the Corticosterone Level in Feathers*

For measuring the corticosterone in feathers, the protocol of Bortolotti et al. (2008) modified by Monclùs et al. (2017) was followed [9,52]. Both studies follow the recommendations of Buchanan and Goldsmith (2004) because each assay technique needs to be fully validated for each new species to get comprehensible results [53].

To synchronize the results, a minimum length of 200 mm per sample was required. Three to five feathers were selected of the same type, thus morphologically identical, and from every individual. Initially, following the protocol, the calamus of the plucked feathers was cut off [9,52,53].

A ball mill (Retsch®, MM200 type with two balls and 25 Hz) was used to mill the feather samples to a particle size of <2 mm. The duration of the process depended on the feather length: small feathers four minutes, larger ones 5 min. Due to the relatively different feather length between individuals, the weight of powder was recorded to the nearest 0.1 mg. A quantity of 1.5 mL of methanol was added to the feather powder of each sample and then mixed in a vortex (Vortex Mixer S0200–230 V-EU; Labnet International, Edison, NJ, USA) at room temperature for 30 min. The subsequent incubation was at 37 °C for 18 h (G24 Environmental Incubation Shaker, New Brunswick Scientific, Edison, NJ, USA). Afterwards, the mixture was centrifuged at 3500× *g* for 15 min (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany). One milliliter of the supernatant was pipetted into an Eppendorf® tube and dried in an oven (Heraeus Function Line T6®, Thermo Fisher Scientific, Waltham, MA, USA) at 38 °C until all liquid had evaporated. After the drying process, the residue was mixed with 0.25 mL of

the buffer solution (containing BSA, NaCl, EDTA and Azide) delivered with the commercial enzyme immunoassay kit (ELISA Neogen® Corporation, Ayr, UK). The composite was shaken in the vortex for one minute and then frozen at −20 °C until analysis. The corticosterone was measured as described by the manufacturer.

### *2.5. Statistical Analysis*

First, the accuracy of the ELISA was proven by creating an intra- and inter-assay coefficient of variation (CV), the linearity of dilution and the measure of fit, $R^2$. The CV was created by pooling ten different samples from ten different individuals. The pool-CV was run three times per assay. Whereas the actual samples had only run the assay once.

An initial sample size of at least 45 animals in each group was chosen to conduct equivalence testing. An equivalence test of means using 45 sampling pairs achieves 90% power at the 5.0% significance level when the true difference between the means is 0.0, the standard deviation of the paired differences is 6.0, and the equivalence limits are −3.0 and +3.0.

The feather samples of this study had a large variance in size (in mm) as well as in mass (in mg). There are studies that use the size while other studies use mass. The feather growth rate is considered to be rather uniform between and within species [54]. Consequently, the hormone exposure is time-dependent and feather corticosterone should be standardized by length [1,9,26,55]. Thus, the chosen unit of this study was pg corticosterone/mm feather.

Two analyses were carried out in order to assess agreement between plucked and cut feathers: the concordance correlation coefficient (CCC) and the Bland–Altman plot. Consequently, the corticosterone values of each method were compared within the same individual.

The CCC is a measurement of precision and accuracy [56]. It ranges from 0 to 1 and reflects the degree to which the two observed values correspond to the 45° line through the origin that indicates perfect agreement [57].

The Pearson's correlation coefficient is also included in the analysis (see Figures 1–3). This value describes a linear relationship between two variables. When its value is 0, there is no linear relationship and the line in the scatterplot is horizontal. However, the value does not describe a cause-and-effect principle and therefore was not considered helpful in this study.

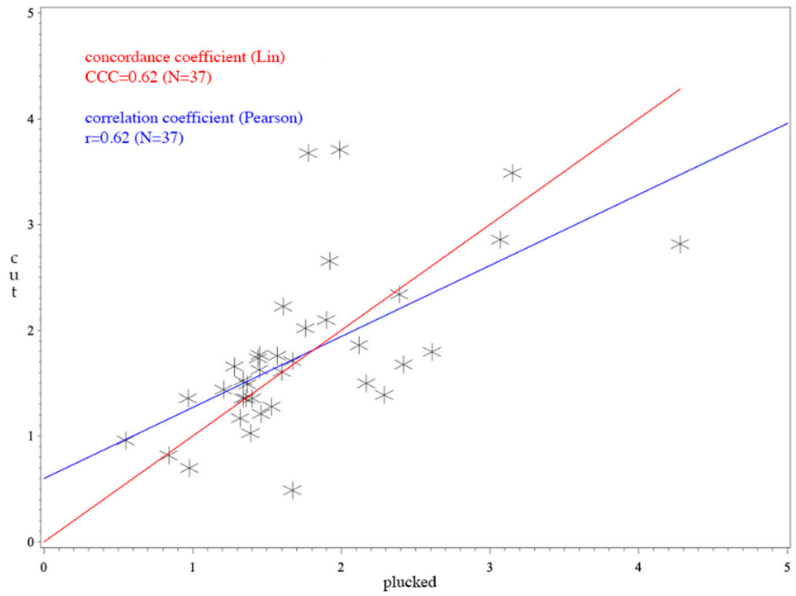


**Figure 1.** Concordance correlation coefficient (CCC) and Pearson's correlation coefficient of *Anas*; *p*-value for Pearson's correlation coefficient: $p = 0.6111 \times 10^{-6}$; the x-axis shows the corticosterone values of plucked feathers. The y-axis represents the values of cut feathers. The red line illustrates the concordance coefficient. The correlation coeffi

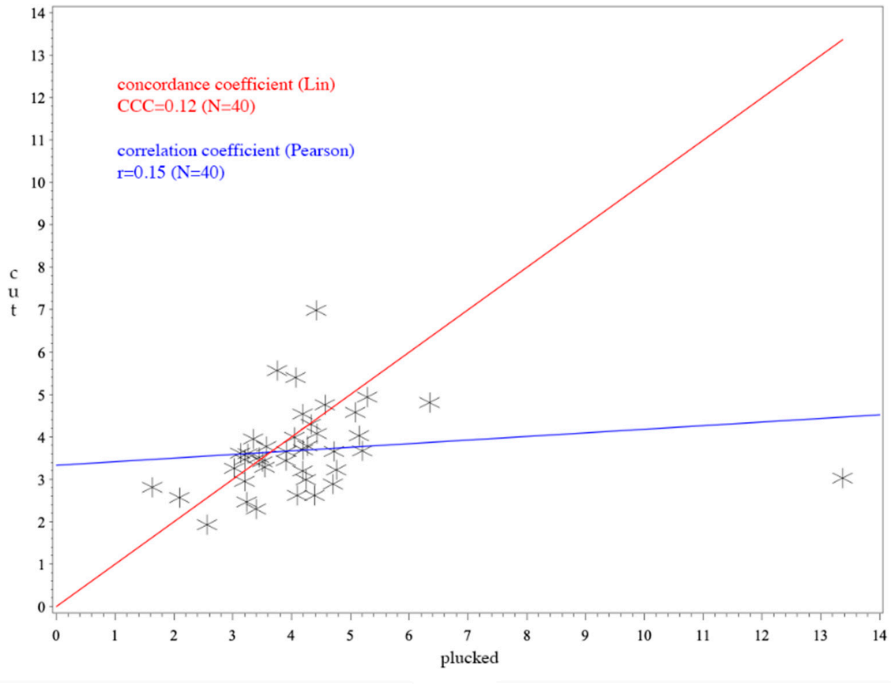


**Figure 2.** CCC and Pearson's correlation coefficient of *Anser*; *p*-value for Pearson's correlation coefficient: $p = 0.4297$; the outlier of 13.37 pg/mm is included. The x-axis shows the corticosterone values of plucked feathers. The y-axis represents the values of cut feathers. The red line illustrates the concordance coefficient. The correlation coefficient is expressed in the blue line.

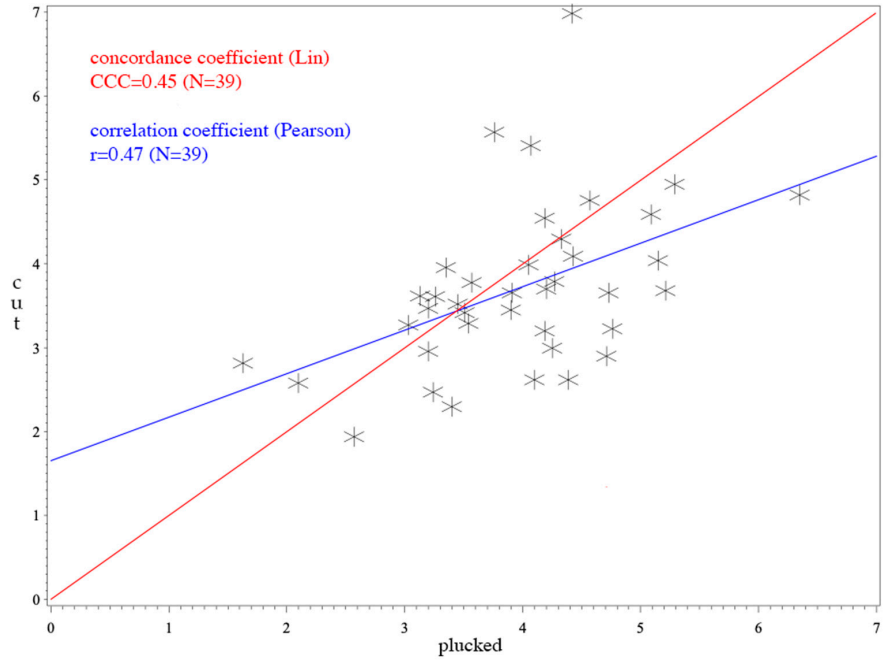


**Figure 3.** CCC and Pearson's correlation coefficient of *Anser*; *p*-value for Pearson's correlation coefficient: $p = 0.0023$; the outlier was excluded which leads to a total number of 39 individuals. The x-axis shows the corticosterone values of plucked feathers. The y-axis represents the values of cut feathers. The red line illustrates the concordance coefficient. The correlation coefficient is expressed in the blue line.

The Bland–Altman plot, also known as the "difference plot", displays the agreement of two measurement techniques [58,59]. The differences between the methods per sampling pair are plotted in a scatter diagram against the respective means of values (see Figures 4 and 5). The middle horizontal

line corresponds to the mean difference. The upper and the lower lines are the 95% confidence interval. In summary, Bland–Altman is a graphical method to assess the relationship between differences and to identify outliers. If the hypothesis is confirmed, the points will lie within the interval and scatter evenly around the centerline. Furthermore, there should be no ascending or descending tendency in the image.

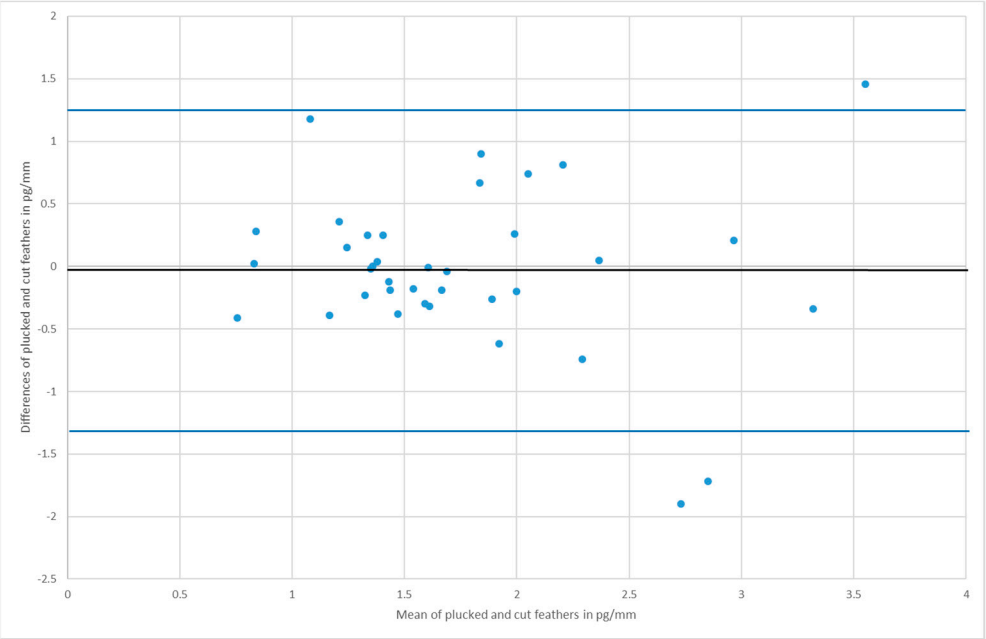


**Figure 4.** Bland–Altman plot of *Anas*; the residual plot displays the differences (y-axis) against the means of values (x-axis). The blue horizontal lines represent the mean difference ± 2 SD (mean = −0.03, SD = 0.65).

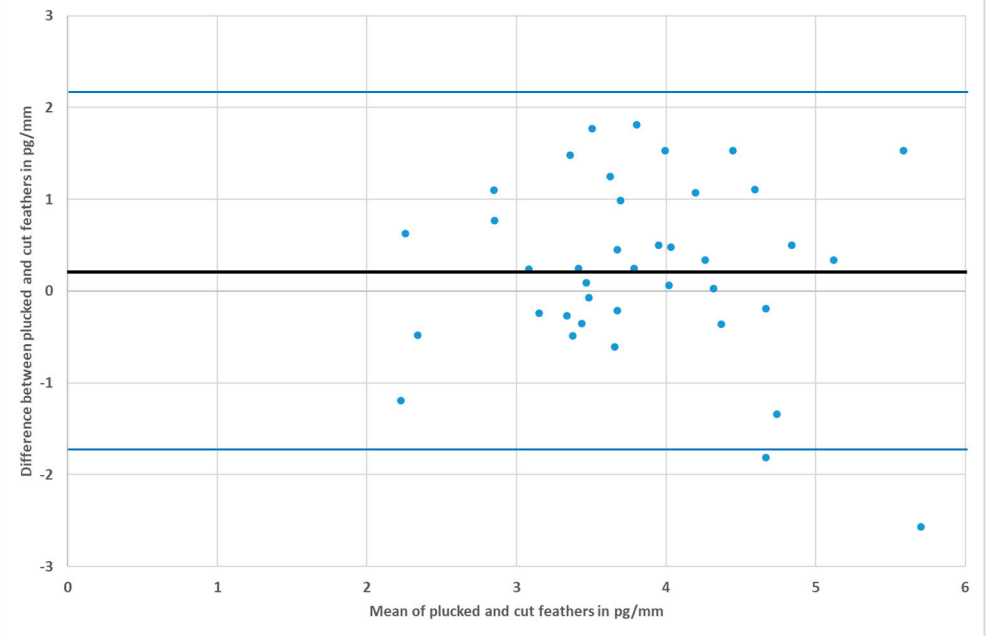


**Figure 5.** Bland–Altman plot of *Anser* without the outlier; the residual plot displays the differences (y-axis) against the means of values (x-axis). The blue horizontal lines represent the mean difference ± 2 SD (mean = 0.25, SD = 0.97).

## 3. Results

In *Anser*, the intra-assay CV amounted to 8.29% and the inter-assay CV to 15.79%. For *Anas*, a value of 8.34% for intra-assay CV and 14.25% for inter-assay CV was calculated. The linearity of dilution was determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of pools with EIA buffer and by calculating the measure of fit $R^2$. The $R^2$-value of *Anser* was 99.72% and that of *Anas* was 99.44%. The values of the spike-and-recovery test amounted to 111.08% ± 8.59% (*Anser*) and 116.40% ± 12.39% (*Anas*).

Due to the sampling conditions during the slaughter process, it was unavoidable that some samples had traces of blood and feces above the feathers; also, a few feathers had blood inside the calamus. This occurred in samples of 20 individuals, six of *Anser* and 14 of *Anas*. The contaminated individuals were excluded from the subsequent analyses [58,59]. This led to a total number of only 40 from *Anser* (initial number of 46) and 37 from *Anas* (initial number of 51).

The description of the feather characteristics and corticosterone levels in a comparison of the two methods is displayed in Table 2.

Since the probability of belonging to the distribution of values was only $5.49 \times 10^{-24}$, one corticosterone value of *Anser* (13.37 pg/mm) was regarded as an outlier and was excluded.

For *Anas*, the CCC was 0.62 (see Figure 1) and for *Anser* it was 0.45 (see Figure 3). The average difference between cut and plucked feathers for *Anas* were −0.0251 with a standard deviation of 0.65 (see Figure 4). This resulted in a 95% confidence interval ranging from −1.2899 to +1.2396. Concerning *Anser*, the mean difference was 0.2544 with a standard deviation of 0.97 and a 95% confidence interval ranging from −1.6529 to +2.1617 (see Figure 5). The total average value of corticosterone of every single sample, whether cut or plucked, was at 1.76 pg/mm for *Anas* and at 3.83 pg/mm for *Anser*.

**Table 2.** Overview of the measured results of 40 geese and 37 ducks in length, weight and corticosterone levels; the contaminated samples were excluded. The values in brackets are those including the outlier.

| | **Length in mm** | | | | **Weight in mg** | | | | **Corticosterone in pg/mg** | | | | **Corticosterone in pg/mm** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Anser*** | | ***Anas*** | | ***Anser*** | | ***Anas*** | | ***Anser*** | | ***Anas*** | | ***Anser*** | | ***Anas*** | |
| | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** | **Plucked** | **Cut** |
| Mean | 222 | 217 | 224 | 225 | 110.3 | 106.0 | 82.0 | 78.7 | 8.01 (8.42) | 7.59 | 4.78 | 5.16 | 3.96 (4.20) | 3.69 | 1.75 | 1.77 |
| Max | 249 | 242 | 251 | 248 | 135.2 | 134.0 | 109.0 | 106.0 | 12.90 (24.49) | 11.54 | 10.88 | 12.24 | 6.35 (13.37) | 6.99 | 4.28 | 3.71 |
| Min | 202 | 201 | 201 | 202 | 74.0 | 56.6 | 53.8 | 52.4 | 3.85 | 4.73 | 1.76 | 1.38 | 1.63 | 1.94 | 0.55 | 0.49 |

## 4. Discussion

The main result of this study shows that no relevant difference in corticosterone was found between feather plucking, the commonly established standard so far, and the alternative method examined of feather cutting. Hereafter, remarkable results of the statistical analysis are pointed out from which conclusions can be drawn.

A standardized sampling region was chosen even though the paper "Carotenoid-based plumage coloration reflects feather corticosterone levels in male House Finches (*Haemorhous mexicanus*)" by Lendvai et al. (2013) showed no difference in hormone levels between tail and breast feathers. However, there are studies on the various levels of absolute hair cortisol in different body regions in chimpanzees (*Pan troglodytes*) and Canada Lynx (*Lynx canadensis*), in which the researchers suggest standardizing the sampling region [60,61]. In addition, it was ensured that only white feathers were analyzed, because Tallo-Parra et al. (2015) describe choosing hair samples homogenous in color and body region in dairy cows [62].

Regarding the sample size, by excluding the contaminated animals, fewer animals are in the statistical evaluation compared to the number (minimum 45) which was calculated at the beginning. Therefore, the desired accuracy could not be maintained and the results should be interpreted with caution. In the future, more animals should be planned as a reserve. Considering the selection, the sampling strategy was indeed rather convenient, but there is no obvious reason for selection bias.

Firstly, the precision of ELISA was assessed by calculating the intra-assay CV from all duplicated samples and the inter-assay CV from samples running different ELISA tests. The specificity was evaluated with the linearity of dilution and the measure of fit, $R^2$. Furthermore, the spike-and-recovery test was applied to assess accuracy, which was calculated by adding a known amount of analyte to different volumes of pure standard cortisol solution. Only one value did not correspond to the required results: the inter-assay CV of *Anas* was just above the 15% reference point, from which a non-optimal inter-assay performance can be concluded [63–65]. However, in summary, the validation tests confirmed that it is possible to detect corticosterone concentrations in feathers of *Anser* and *Anas* with an acceptable repeatability and reliability by using ELISA.

With the chosen unit pg/mm, the corticosterone values were not as widely distributed as those expressed in pg/mg (Table 2). Due to this lower dispersion, we considered the values expressed in pg/mm to be more precise, considering the time-dependent aspect, and more appropriate than those in pg/mg [9].

Comparing the two different sampling methods, plucking resulted in values 0.27 pg/mm higher in *Anser* (7.1% of the total average value), whereas in *Anas* the cut feathers showed values only 0.02 pg/mm higher (0.6% of the total average value). In consequence, a statement that plucked feathers produce generally higher or lower values could not be supported. With a comparison of the mean difference between methods of 0.03 pg/mm (*Anas*) vs. 0.25 pg/mm (*Anser*) and the respective standard variations of 0.65 (*Anas*) and 0.97 (*Anser*), it becomes clear that there is no systematic difference between the methods that would lead to a systematic under- or overestimation. Hence, no general conclusions can be drawn about the effects on the corticosterone levels of one method or the other, and the differences between the methods can be said to be negligible.

A value of CCC under 0.9 is regarded poor, 0.9–0.95 moderate, 0.95–0.99 substantial and above 0.99 almost perfect [56]. Thus, the CCCs observed have to be regarded as poor in total. The CCC of *Anser* (Figure 3) was not as high as that of *Anas* (Figure 1). The higher value of CCC (0.62) implies a better agreement in *Anas*. This cocanuld also be observed in the Bland–Altman plot. However, it must be considered that CCC mainly assesses methodological differences using an identical sample. This is not the case in our study because the feathers were indeed from the same individual, but they were biologically different between the two sampling methods. Nevertheless, in terms of comparison with the mean values, the differences can be considered marginal.

In total, *Anser* produced a higher amount of corticosterone (3.82 pg/mm), whereas *Anas* had an average of 1.76 pg/mm. This was also reflected in the mean differences of the single values: 0.25 pg/mm

for *Anser* and −0.03 for *Anas*. Additionally, *Anas* had a substantially lower standard deviation. Due to the larger differences of *Anser* and the more similar values of *Anas*, there is an overall better match of this study regarding *Anas*.

However, looking at the plot of *Anas* (Figure 4), three values were beyond the limits. Nevertheless, the measured results scattered more or less perfectly around the mean. Although the agreement in *Anser* was not as good as that in *Anas*, the Bland–Altman plot showed a fairly good agreement (Figure 5).

In conclusion, the agreement between plucked and cut feathers was rather poor, mainly concerning the CCC. However, taking into consideration the large variation in corticosterone levels within species, especially in the group of geese, and that there was no greater difference between the methods regarding all subjects, or no general trend of one group having higher levels than the other, it can be assumed that the poor agreement is rather due to general data variation. In addition, in contrast to the CCC, the agreement in the plot can be interpreted as more appropriate. Thus, cut feathers seem to be as suitable as plucked feathers.

Nevertheless, it must be added that the large variation of values within a species, as mentioned above, may also be explained with the following drawbacks of this study. Although the sampled feathers were standardized as far as possible (interscapular region, same color, same morphology, similar total length), the standardization is not as optimal as comparing a precisely identified feather between the subjects, for example the first wing feather. However, since the study should also serve as a basis for projects with wild birds, the sampling of a flight or tail feathers would be considered very critical because of their influence on the ability of flight. Therefore, we have chosen the sampling site between the shoulders. However, in the future, several cut feather samples from one individual should have to be analyzed in order to better assess the individual variation of the corticosterone levels. The same applies to the plucking method. Only then could the variation within a method be compared with the variation between methods and thus, draw conclusions about the possible negligibility of the discrepancy between methods. Feather corticosterone has been validated for tracking stress in several studies [1,9,26,66]. The purpose of this study was to test agreement between cut and plucked feathers within the same animals. The animals used were assumed to be not chronically stressed. To assess the relevance of the within-individual variation (median 15.6% for *Anser*, 17.8% for *Anas*), this and the maximum values (6.99 pg/mm in *Anser*, 4.28 pg/mm in *Anas*) must be compared to values from animals kept under different husbandry conditions. Since there are no values from stressed *Anser* and stressed *Anas* available in the literature, we discuss results of a study performed on Greater Flamingos for an estimation [48]. Stress was expected in one group due to repeated attacks by cranes. This group had a mean corticosterone level of 15.96 pg/mm and a maximum value of 20.93 pg/mm. The group with the smallest corticosterone values had a mean of 5.62 pg/mm. Although the absolute values cannot be compared between species, it seems that the differences between stressed and unstressed birds are much greater than the observed differences of 15–18%. This leads to the conclusion that although the statistical agreement between cut and plucked feathers was not satisfactory, the variation is not biologically relevant because all values were in a range below those expected in stressed animals. In addition, regarding this study, through the large standard deviation in corticosterone itself, the value alone allows us no general statement about the individuals' stress. Therefore, corticosterone values should always be interpreted in combination with additional data, for example behavioral observations, or after repeated measurements.

## 5. Conclusions

The less-invasive method of cutting feathers seems to work as an alternative to plucked feather samples. Nevertheless, we refer to 'less-invasive', because it should not be forgotten that the birds must also be caught and restrained while cutting the feathers. This method should therefore only be used, if possible, if the bird is caught for other purposes anyway. However, the concordance, especially regarding the CCC values, was rather poor. Therefore, it is very important to encourage further studies

to underpin the significance of this project. In future projects we would like to cut feathers from live birds instead of plucking them and thus make a contribution to the animal in the sense of refinement. Additionally, we hope that further studies can confirm this result in other bird species. Furthermore, other factors influencing the corticosterone level should be evaluated; for example, gender, as this factor was not taken into account in this study. Once a larger set of data has been established and cutting feathers is a suitable alternative, the influence of different husbandry conditions should play a major role in subsequent projects. Concerning this topic, ongoing studies, as well as currently published studies, focus on the evaluation of deflighting zoo birds, the comparison between different husbandries and the welfare implications. These deal with the species Greater Flamingo (*Phoenicopterus roseus*) [48] and Great White Pelican (*Pelecanus onocrotalus*) (ongoing study). The planning, structure and realization of research projects compared to these could also be expanded and simplified with this non-invasive sampling method.

Further studies to confirm the results of this study are planned on wild species as well as young birds.

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## 4 Comparison of Two Different Feather Sampling Methods to Measure Corticosterone in Wild Greater Flamingos (*Phoenicopterus roseus*) and Wild Mallard Ducks (*Anas platyrhynchos)*

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Abstract:

This research project had the aim to validate the possible alternative and less-painful sampling method of cutting feathers close to the skin instead of plucking them for subsequent feather corticosterone analysis, confirming recently-published results for other species in captivity.

Analyzing CORTf is often used in animal welfare studies in combination with behavioral monitoring. The background of this idea was to act in the sense of animal welfare and reduce the burden of animal studies according to the 3-R-Principle (Replacement, Reduction, and Refinement) by refining procedures. To confirm the hypothesis that the sampling method itself has no influence on CORTf levels measured, plucked and cut samples of the respective bird were collected. Birds of two wild species were used: the Mallard (*Anas platyrhynchos*) and the Greater Flamingo (*Phoenicopterus roseus*). The CORTf was measured by using an enzyme-linked immunosorbent assay (ELISA). The determined values were inspected for their mean values, standard deviation (SD), and average differences. Afterwards, the CORTf levels of both species were compared, according to the sampling method, with the concordance correlation coefficient (CCC). In the Bland-Altman (BA) plot the differences of the methods were displayed against the mean values. Additionally, sex, as a possible factor influencing CORTf, was analyzed using the Mann-Whitney U test. The values of CCC showed poor agreement in the comparability of the two methods, whereas the concordance of the BA plot was decent. The average differences between the methods were marginal for both species (Mallards: −0.16 pg/mm, Flamingos −0.13 pg/mm). In summary, all anomalies or differences between the methods were negligible. Therefore, the alternative sampling method seems to be as suitable as the common standard method. No significant difference was found between females and males. Nevertheless, our results suggest that CORTf should not be interpreted in just considering the values themselves, but the results they should be analyzed in the context of a wider set of parameters. Hence, further studies are encouraged to create a larger data pool.

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# Comparison of Two Different Feather Sampling Methods to Measure Corticosterone in Wild Greater Flamingos (*Phoenicopterus roseus*) and Wild Mallards (*Anas platyrhynchos*)

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**Simple Summary:** The common standard sampling method to determine corticosterone in feathers (CORTf) is to pluck them from the bird's skin. This procedure is considered to be painful, and the animals have to be caught and fixated firmly. Therefore, an animal experiment approval is required according to European and German legislation. In this study, we compared two methods: plucking vs. cutting feathers. The aim was to confirm the validation of an alternative less-invasive sampling technique. The specimens of this project were wild adult Mallards (Germany) and wild 1st-calender-year juvenile Greater Flamingos (Spain). In summary, there were no significant differences between the methods in terms of corticosterone results for both species. Additionally, no differences were found in CORTf between females and males of both species. In conclusion, these findings underline the suitability of cutting feathers as a sampling method for the determination of CORTf levels.

**Abstract:** This research project had the aim to validate the possible alternative and less-painful sampling method of cutting feathers close to the skin instead of plucking them for subsequent feather corticosterone analysis, confirming recently-published results for other species in captivity. Analyzing CORTf is often used in animal welfare studies in combination with behavioral monitoring. The background of this idea was to act in the sense of animal welfare and reduce the burden of animal studies according to the 3-R-Principle (Replacement, Reduction, and Refinement) by refining procedures. To confirm the hypothesis that the sampling method itself has no influence on CORTf levels measured, plucked and cut samples of the respective bird were collected. Birds of two wild species were used: the Mallard (*Anas platyrhynchos*) and the Greater Flamingo (*Phoenicopterus roseus*). The CORTf was measured by using an enzyme-linked immunosorbent assay (ELISA). The determined values were inspected for their mean values, standard deviation (SD), and average differences. Afterwards, the CORTf levels of both species were compared, according to the sampling method, with the concordance correlation coefficient (CCC). In the Bland-Altman (BA) plot the differences of the methods were displayed against the mean values. Additionally, sex, as a possible factor influencing CORTf, was analyzed using the Mann-Whitney U test. The values of CCC showed poor agreement in the comparability of the two methods, whereas the concordance of the BA plot was decent. The average differences between the methods were marginal for both species (Mallards:

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−0.16 pg/mm, Flamingos −0.13 pg/mm). In summary, all anomalies or differences between the methods were negligible. Therefore, the alternative sampling method seems to be as suitable as the common standard method. No significant difference was found between females and males. Nevertheless, our results suggest that CORTf should not be interpreted in just considering the values themselves, but the results they should be analyzed in the context of a wider set of parameters. Hence, further studies are encouraged to create a larger data pool.

**Keywords:** feather corticosterone; Mallard; Greater Flamingo; wild birds; comparative study; cut feathers; plucked feathers; less invasive; animal welfare; refinement; sex comparison

## 1. Introduction

To assess the well-being of a bird, different approaches have been developed so far. One possible way to evaluate the animal's welfare is to combine behavioral observations with corticosterone (CORT) measurements. Corticosterone is the main glucocorticoid (GC) in birds, reptiles, amphibians, and rodents, whereas in other mammals and fish, it is cortisol [1,2]. An increasing level of GC is especially associated to the presence of stress stimuli [3,4]. In the case of a sudden stimulus, the individual first experiences an increased secretion of catecholamines, followed by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of GC into the bloodstream (fight-or-flight response) [4,5]. This response has diverse effects on the bird's physiology (e.g., increasing heartrate and blood pressure) and helps to reduce risk and ensure survival [4,6]. However, GC cannot only be seen as a 'stress-hormone' [5]. It is released in baseline concentrations, as well as in various concentrations which can be related to different internal and external influencing factors [2]. Such influencing factors could be age, sex, or body condition, and it varies with climate or season, for instance [2]. To interpret the GC levels, it is important to retrace the actions of the HPA axis and understand the reactions to chronic stress. Sapolsky et al. have described the GC impacts as preparative, stimulating, suppressive, or permissive to maintain homeostasis in challenging occasions [4]. In other studies, the concept of allostasis has been used as a basis, a situation in which stability is preserved through change [7,8]; Romero et al. have modified it in their 'reactive scope model' (integrating homeostasis, allostasis, and stress) [9].

Nowadays, measuring corticosterone in feathers (CORTf) is an increasingly used and validated method to evaluate stress levels [3]. The CORTf enables the researcher to obtain a retrospective view of the activity of the bird's HPA axis during the time of feather growth [3,10]. In the molting period, blood circulates in the feather follicles, and CORT diffuses into the feather. As described in a study by Bortolotti et al. and then confirmed in other studies, increased plasma CORT levels during feather growth result in elevated CORTf levels [11–14]. To determine CORTf, the feathers are sampled by plucking them from live or dead birds or even by collecting the ones dropped by molting birds [10,11,13,15]. Depending on the scientific context, the aspect of a long-term and retrospective view on the activity of the HPA axis could be a clear benefit compared to other corticosterone measurement methods. For good sample quality, feather growth should be completed at the time of sampling, and the feather should be dry and clean; only then, the CORTf level can be related to the time of feather growth [10]. An advantage of CORTf measurement is the storage of feathers. Because feathers are a stable matrix, except of storing them dry and clean, there they require no further treatment [3,16–19]. This enables longer storage, and thus, e.g., samples of rare species or wild birds can be collected when the opportunity arises and analyzed at a later time if needed.

In a previously published study, we addressed the issue of validating an alternative method, cutting feathers, in captive geese and ducks instead of feather plucking, the common sampling method, for CORTf measurement [20]. In addition, we evaluated those results in this study with wild birds and dealt with the influencing factor 'sex'. The

reason to validate an alternative method was that the plucking of a feather is regarded painful for the bird because it results in reactions of the body, e.g., increasing heart rate and blood pressure or behavioral changes, which may be associated with pain [21–24]. Therefore, to examine CORTf in plucked feathers of living birds, an animal experiment application is needed in Germany and many other EU countries. To act in the sense of animal welfare and according to the EU Directive 2010/63/EU and 3-R Principle, with the aim to reduce animal distress, the idea of the alternative and less-invasive method of feather-cutting arose [25,26], where the feather can be cut off near the bird's skin. As in a previous study [20], the region between the shoulders was chosen as sampling site in this study. The localization was perfect for fast and safe sampling and had no impact on the bird's flight ability. This sampling region has already been used in other research projects [19,27,28]. An additional point in favor of the alternative cutting technique is that in the process of CORTf determination, the calamus of the feather is first cut off before the feather is analyzed in more detail (e.g., measurement of length and weight) [11,16,28,29]. Consequently, plucking the feather is not necessary for the following analysis.

In this research project, two species of wild animals were examined: adult Mallards (*Anas platyrhynchos*) and 1st-calender-year juvenile Greater Flamingos (*Phoenicopterus roseus*). The wild Mallards were shot for hunting reasons, whereby no animal experiment was needed. Regarding the flamingos, through the project "Anillamiento de flamencos", we had the opportunity to sample wild Greater Flamingos [30]. The application for the animal experiment has already been approved by the competent authorities in Spain [31,32]. With these chosen wild species we had the chance to discuss the comparability of CORTf to results of two other studies. Drawing a comparison of the wild ducks of this study with the Mulard Ducks (*Anas sterilis resp. Cairina moschata* × *Anas platyrhynchos*) of a conventional poultry farm was possible [20]. Additionally, the availability of feathers from juvenile flamingos allowed a comparison with recent study results on adults [27]. The German Veterinary Association for Animal Protection (Tierärztliche Vereinigung für Tierschutz—TVT) investigates and evaluates husbandries of certain captive bird species ethologically and physiologically in more detail [33]. Both orders of Anseriformes and Phoenopteriformes have been listed by the TVT to encourage science-based animal welfare assessments.

The aim of this study was to validate cutting as a less-invasive method to determine CORTf in wild bird species and to confirm existing results that suggest the sampling method itself has no effect on the measured CORTf levels [20]. In addition, a comparison of CORTf between female and male birds was examined to consider sex as a possible influencing factor. Furthermore, an outlook was formulated on the comparability of data between captive and wild birds of this study.

## 2. Materials and Methods

### *2.1. Sampling Protocol*

The feathers were plucked and cut close to the skin between the shoulders, as described before [20,27,34–36]. The wearing of gloves while sampling avoided any contamination (e.g., sweat), and additionally, the researcher ensured that the feather quills were dry and free of any biological contamination (e.g., blood, feces). All samples had the same morphology (cover feathers) and a similar total length (300–400 mm). In addition, to obtain a better agreement in the comparison of CORTf levels, the selected feathers had the same color within the species (flamingos with gray feathers, Mallards with brown feathers) [10,37–39]. A minimum sample size of 45 animals of each species was determined biometrically, resulting in an equivalence test of means with 90% power and 5.0% significance level, whereas the true difference between the means was 0.0 [20]. The samples were stored in paper envelops at room temperature until laboratory examination. Another possible influencing factor of CORTf levels was included: the samples were differentiated according to the individual's sex [3,11,36,37,40]. An ethical review did not need to be approved in this study because the ducks were hunted and the sampling of flamingos was conducted in the context of an already approved project in Spain [30].

### *2.2. Specimens*

First, it should be made clear that the most accurate information possible on a bird's habits, molting status, and biological data is crucial for interpreting CORTf [3,17]. However, since this study was based on wild birds, no individual data were available. Therefore, the focus was placed on the knowledge about the populations of the respective species.

#### 2.2.1. Mallard (*Anas platyrhynchos*)

The Mallard is assigned to the genus group of dabbling ducks (*Anatinae*) [41,42]. They are widespread and common in the Eurasian and North American regions as well as in parts of North Africa [43]. The duck species sampled in this study is the native and most common of its species in Germany: *Anas platyrhynchos platyrhynchos*. This species is not water-bound, but they stay on the water after sunrise for resting and dabbling [42]. Their habitat is near to water, such as lakes, small ponds, rivers, and in winter also on the seashore [44]. One of the peculiarities of ducks is the beak structure, which is similar to that of the flamingo. The lamellae of the beak and the tongue create a kind of sieve apparatus that filters food such as small animals, insects, or green plant parts from the water [41]. In general, they are omnivores. The courtship of the ducks occurs in fall, passes into an engagement period, the fixed mating in January/February, and, finally, the egg-laying period in March, followed by a breeding period of around 28 days [42]. The ducklings are nidifugous, covered in down, but are able to eat, swim, and dive [45]. They reach flight maturity in summer at the same time as the adult drakes after their large plumage molt in June or July [41]. The general molt of the duck takes place later, at the end of July and the beginning of August [42]. The small-feather molting occurs twice a year [41]. In October, the drake changes to its splendor feather dress for the mating season, which it keeps until spring [42]. In this period, the animals show apparent sexual dimorphism whereby they are distinguishable visibly.

The hunting season for Mallards in Germany is legally set from 1 September to 15 January [42,44]. The sampling took place on several days from 27 October until 28 December 2019 after the specimens were shot in hunting manners in the district "Nürnberger Land", east of Nuremberg. Both sexes and the ducklings of the same year are capable of flight at this time, therefore it is important to distinguish them on the basis of certain characteristics: The drakes wear their unique plumage for the mating season, which made it possible to differentiate the sexes visibly. In Mallards, juveniles can be recognized by their darker, gray-greenish feet and reddish horn-colored bill, whereas adults have light yellow feet, which even turn to orange-red from the second year of life [42].

In total 47 Mallards, 22 females and 25 males (differentiated via visible sexual dimorphism), were sampled. All specimens were identified as adults (via visible characteristics). A minimum of 10 feathers were sampled by plucking as well as by cutting them closely to the skin from each duck.

#### 2.2.2. Greater Flamingo (*Phoenicopterus roseus*)

The Greater Flamingo is classified in the order of *Phoenicopteriformes* [41]; its distribution area is in Africa, Asia, and Europe [46]. The flamingos of this study were located in southwest Spain in Marismas del Odiel, a nature reserve nearby Huelva.

Flamingos live together in large flocks at salt lakes, salt pans, coastal brackish waters, or similar ecosystems [41]. In this habitat, there is often mass reproduction of the brine shrimp (*Artemia salina*), which, together with copepods and insect larvae, is on the food list of flamingoes, followed by algae and diatoms [41,45]. Similar to the Mallards described above, the flamingo has a unique beak that is used in combination with the tongue to filter these brine shrimp; however, the beak is inverted during filtering, so the mandible is uppermost [45]. It is very difficult to differentiate male and female Greater Flamingos [41]. In Spain, the breeding period usually lasts from April until June [47]. The classification of the study specimens in terms of their age was done following the definitions of Grzimek's Animal Life Encyclopedia [41]: egg incubation = 27–31 days; precocial = 4–7 days after

hatching; white-gray down, red feet, and red, straight beak = newborn until 7–10 days; black feet and black, straight beak = after age of 7–10 days; molting in second gray down and bill bending = age of 2–3 weeks; small plumage in shoulder area = age of 4 weeks; flight ability = age of 70 days; juvenile plumage = gray-brown; molting in pale plumage = age of 9–18 months; full coloration = age of 3–4 years.

The specimens were sampled in combination with the project "Anillamiento de flamencos" on 19 July 2019. This project is organized and supported by several Spanish and European organizations and has been existing since 1986 [31]. Sampling takes place annually in two natural reserves in Andalusia, Marismas del Odiel, and Fuente de Piedra, where large colonies of Greater Flamingos breed. In this operation, the colony was slowly circled in the early morning hours by many volunteers. The adult flamingos flew away, leaving the chicks that were still unable to fly. After caging them, each chick was individually captured, weighed, photographically documented, sampled for blood and feathers, ringed, and finally released back into the wild. These annual examinations and documentations serve to monitor the colony and its reproductive success [32]. With the official approval of the whole project by the Spanish authorities, feather sampling on living flamingos was allowed for our study. The chicks already had a curved beak and were still in a gray plumage, which, however, had molted to the first small plumage in the shoulder area. Considering the Spanish breeding season, the age was around 4 weeks to less than around 10 weeks old, since the flamingos were still unable to fly.

A total number of 46 juvenile flamingos were sampled. Per sampling technique (cutting and plucking), 7 to 10 feathers were collected. Since the juvenile birds could not be distinguished externally with certainty in terms of sex, feathers had to be examined separately for each bird by DNA analysis (laboratory of 'Tauros Diagnostik—Veterinärmedizinische Analysen'). It turned out that 26 female and 20 male flamingos were sampled.

### *2.3. Analysis of CORTf*

The measurement of CORTf was performed with an ELISA kit. For feather processing, we strictly followed the protocol of Bortolotti et al. (2008) and the modified version of Monclús et al. (2017) [11,16]. To obtain accurate results, it is additionally recommended to perform a new assay validation for each species under investigation [29].

First, several feathers from both Mallards and flamingos were tested to determine the minimum total feather length needed to produce sufficient powder for the measurement and the optimal time in the ball mill to obtain a fine and uniform powder. For both species, a length of 300 to 400 mm was determined per sampling method, which resulted in 9 to 10 feathers required for each Mallard and 7 to 10 feathers for each Greater Flamingo. In addition, to avoid confounding factors, the sampled feathers needed to be from the same type and, thus, morphologically identical. The first step of further processing is cutting off the calamus in the plucked samples [11,16,29]. Additionally, it was ensured that the calamus was completely off at the cut feather samples; otherwise, it was touched up. The feather samples were weighed to the nearest of 0.1 mg, and subsequently, the ball mill (Retsch®, MM200 type with two balls and 25 Hz, Germany) ground the samples to a particle size of ~10 µm for 4 min for the relatively small feathers. The weight of the powder was compared to the starting weight to check for major loss. Next, the feather powder of each sample was mixed with 1.5 mL of methanol in a vortex (Vortex Mixer S0200–230 V-EU; Labnet International, Edison, NJ, USA) at room temperature for 30 min, and the mixture was incubated at 37 °C for 18 h in a G24 Environmental Incubation Shaker (New Brunswick Scientific, Edison, NJ, USA). Hereafter, the sample was centrifuged at 3500× *g* for 15 min (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany). From the resulting supernatant, 1 mL was pipetted into an Eppendorf® tube, which was then placed in an oven (Heraeus Function Line T6®, Thermo Fisher Scientific, Waltham, MA, USA) at 38 °C until all the liquid had dried. The residue was dissolved in 0.25 mL of the buffer solution (containing BSA, NaCl, EDTA and Azide) of the commercial enzyme immunoassay kit (ELISA Neogen® Corporation, Ayr, UK) and mixed in the vortex for

1 min. If the sample was not immediately used in the ELISA for CORT measurement, it was frozen at −20 °C until analysis.

### *2.4. Statistical Analysis*

The precision of the ELISA was verified by the inter- and intra-assay coefficient of variation (CV) [48–50]. For measuring the CV, a pool of 10 different samples of 10 different individuals was created to run the assay in triplicates, whereas the actual samples were analyzed in single runs. We evaluated the intra- and inter-assay CV using a pool with both species, resulting in an intra- and inter-assay CV of 9.44 and 11.96%, respectively. The same kit and protocol have previously been validated separately for both species, with similar results [20,27].

All samples were in good condition and not contaminated with blood or feces; therefore, all CORTf results could be used for analysis [50]. Corticosterone values were expressed as pg CORTf/mm feather length. Since it can be assumed that the feather growth rate is uniform in morphologically similar feathers of one individual and sampling region, hormone exposure is time-dependent and should therefore be specified by length [3,11,17,18,51].

Data were collected in MS Excel® version 2016 and analyzed with IBM SPSS v. 25.

To assess the agreement between plucked and cut feathers, the CORTf of each method was compared within the same individual. For this, Lin's concordance correlation coefficient (CCC) and the Bland-Altman plot (BA-plot) were used for analysis.

The figure of the CCC presents the degree of dependence of the observed values to the 45° line through the origin, indicating perfect agreement (see Figures 1 and 2) [52]. Consequently, the CCC measures the precision and accuracy of a test method [53].

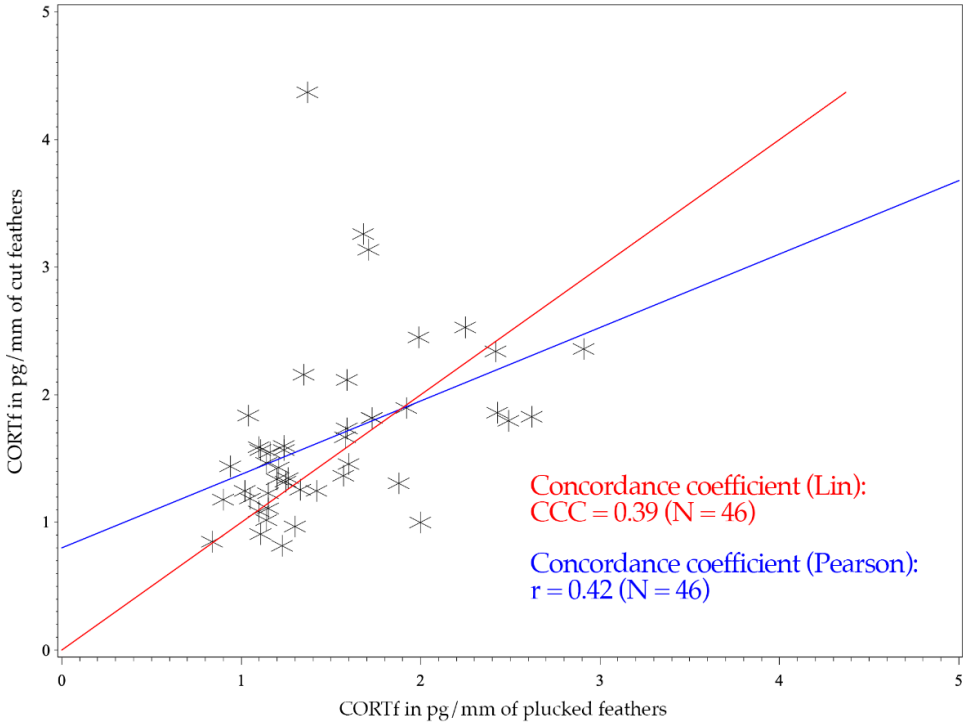


**Figure 1.** CCC and Pearson's concordance coefficients of 46 Mallards (*Anas platyrhynchos*). The *x*-axis shows the CORTf values of plucked feathers in pg/mm, and the *y*-axis represents the values of cut feathers in pg/mm. The red line illustrates Lin's concordance coefficient. Pearson's correlation coefficient is expressed with the blue line.

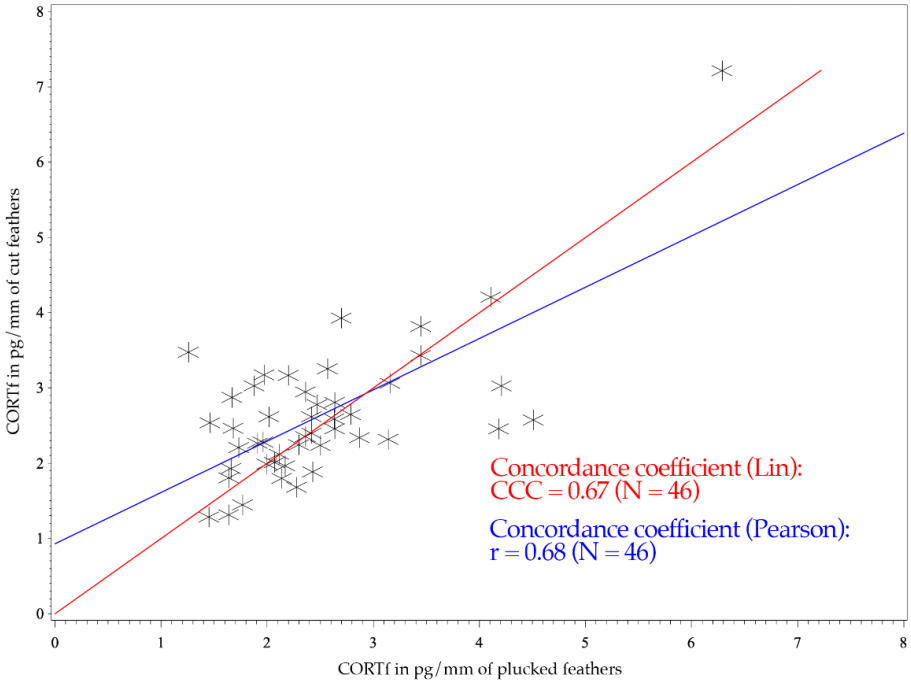


**Figure 2.** CCC and Pearson's concordance coefficients of 46 Greater Flamingos (*Phoenicopterus roseus*). The *x*-axis shows the CORTf values of plucked feathers in pg/mm, and the *y*-axis represents the values of cut feathers in pg/mm. The red line illustrates Lin's concordance coefficient. Pearson's correlation coefficient is expressed with the blue line.

Regarding the figures of the BA-plot, the difference between CORTf of the two sampling techniques of one individual were set in relation with the respective means of values (see Figures 3 and 4). Each point represents a pair of values in the graph; the centerline is the mean difference, complemented by a 95% confidence interval. The image of points should not show an ascending or descending tendency, but scatter around the mean difference. Subsequently, the BA-plot assess the agreement between the two sampling methods and displays differences and outliers [20,54,55].

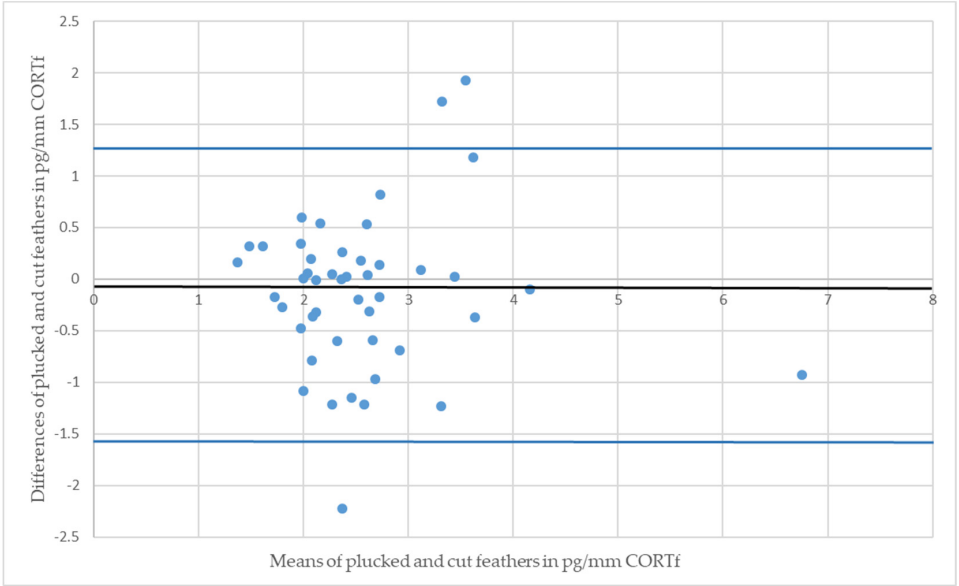


**Figure 3.** Bland-Altman plot of Mallards (*Anas platyrhynchos*); the residual plot displays the differences (*y*-axis) against the means of values (*x*-axis) of CORTf in the two sampling methods. The blue horizontal lines represent the mean difference ± 2 SD (mean = −0.16, SD = 0.65).

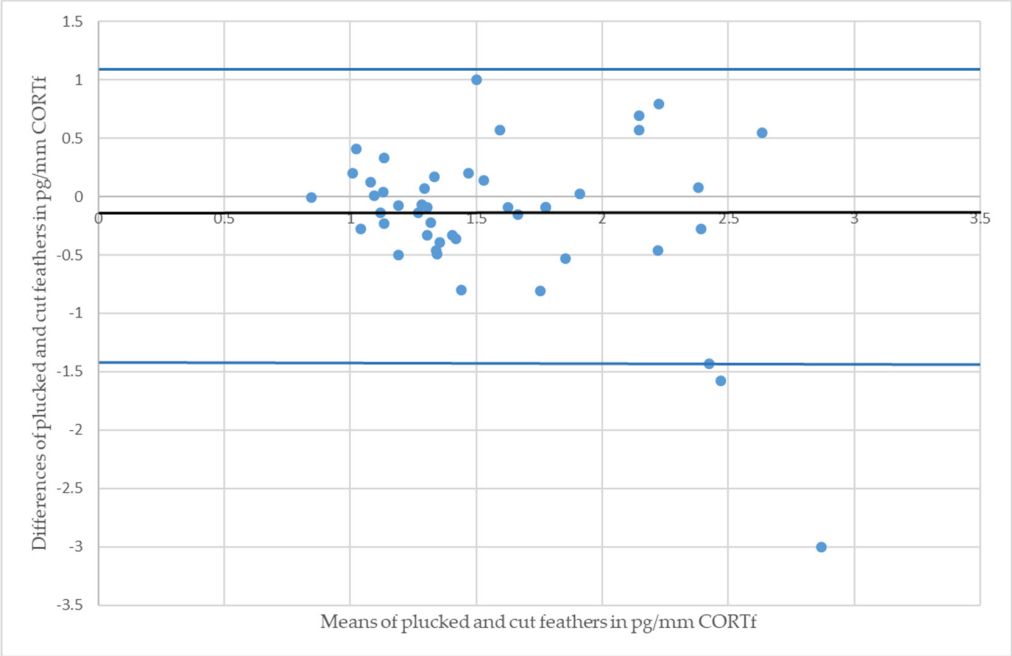


**Figure 4.** Bland-Altman plot of Greater Flamingos (*Phoenicopterus roseus*); the residual plot displays the differences (*y*-axis) against the means of values (*x*-axis) of CORTf in the two sampling methods. The blue horizontal lines represent the mean difference ± 2 SD (mean = −0.13, SD = 0.76).

In similar studies with Common Redpolls (*Acanthis flammea*) and Snow Petrels (*Pagodroma nivea*), a difference in plasma CORT level between the sexes was found [37,40]. Therefore, we investigated the relationship between sex and CORTf level using the Mann-Whitney U Test. This test was applied because the data were not normally distributed [56]. Results are displayed in a box-plot figure (see Figures 5 and 6).

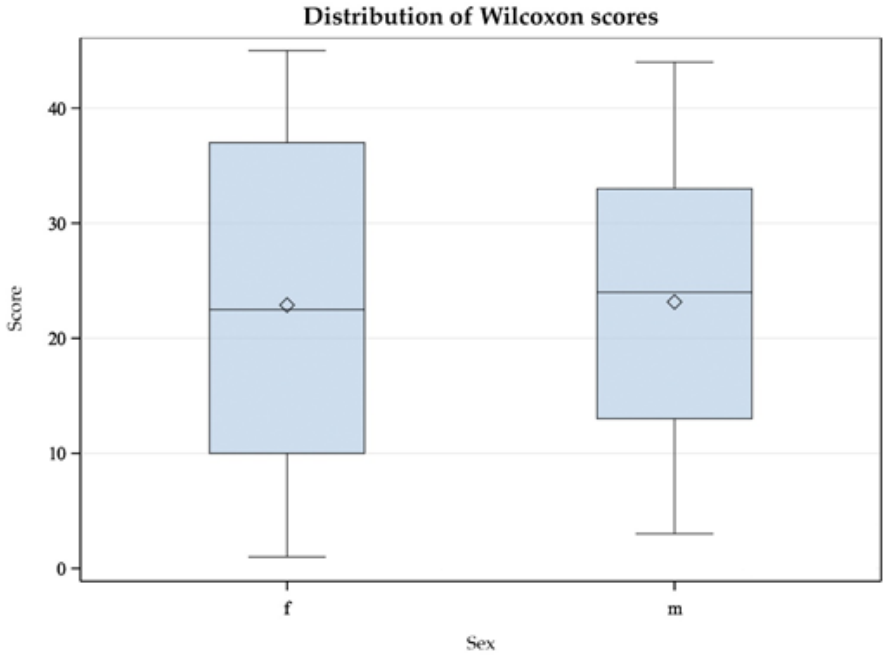


**Figure 5.** Mann-Whitney U test with double-sided t-approximation plotting the differences of female (f) and male (m) Mallards (*Anas platyrhynchos*) in their CORTf levels.

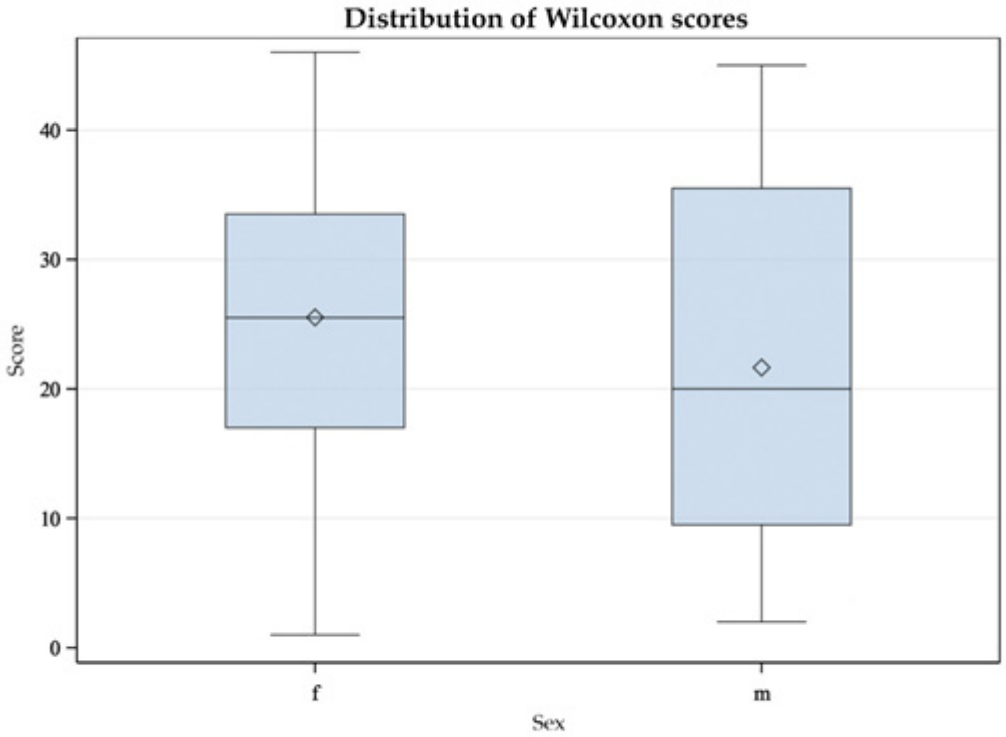


**Figure 6.** Mann-Whitney U test with double sided t-approximation plotting the differences between female (f) and male (m) Greater Flamingos (*Phoenicopterus roseus*) in their CORTf levels.

## 3. Results

In the following analysis, we had a total number of 47 Mallards, with 22 females and 25 males. The CORTf value of one male was identified as an outlier (8.10 pg/mm in the cut samples), and this individual was therefore excluded from analysis (see Table 1). This resulted in a total of 46 ducks. This number was equal to the number of sampled flamingos ($n$ = 46), although we examined 26 females and 20 males (see Table 2). In the results for the Greater Flamingos, one value did not correspond to the specified norm: the minimum total length of plucked feathers was 236 mm. Thus, the value fell below the requirement for total length. Equally notable is the significantly higher (>2 pg/mm) maximum value of CORTf in pg/mm in female flamingos compared to males, irrespective of the sampling method. In total, the feather samples of the Mallards compared to the Greater Flamingos had a higher total length, by 18 mm (plucked) and 14 mm (cut), and an overall lower CORTf level, by 1.01 pg/mm (plucked) and 0.97 pg/mm(cut), respectively. An overview of the CORTf levels of both species, their other feather characteristics (length, weight), and a comparison of the results between the two sampling techniques, as well as between sexes, is displayed in Tables 1 and 2.

The Mallards had a CCC of 0.39 and a Pearson's concordance coefficient of r = 0.42 (see Figure 1); whereas the measurements of Greater Flamingos resulted in a CCC value of 0.67 and a Pearson's concordance coefficient of r = 0.68 (see Figure 2). Considering the BA-plot, Mallards had an average difference of all CORTf levels in pg/mm between the two methods of −0.16. They produced a standard deviation of 0.65, with a 95%-confidence interval ranging from +1.12 to −1.44. The CORTf values of Greater Flamingos created an average difference of −0.13, with a standard deviation of 0.76, resulting in a 95%-confidence interval from +1.36 to −1.61.

The differences in CORTf levels between female and male Mallards were not significant, with $Z$ = 0.97, $p$ = 0.3384 (see Figure 5). There was also no statistically significant difference in CORTf between sexes in Greater Flamingos, with $Z$ = 0.06, $p$ = 0.9544 (see Figure 6).

**Table 1.** Overview of the measured results (total feather length, weight of feathers, and CORTf) of 46 (47) Mallard (*Anas platyrhynchos*) compared between sexes, with 22 females and 24 (25) males, and sampling method (plucked vs. cut); 9 to 10 feathers were used for analysis. The values in brackets indicate the results including the outlier.

| Sex | | Total Length of Feathers (in mm) | | Weight of Feathers (in mg) | | CORTf (in pg/mm) | | CORTf (in pg/mg) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plucked | Cut | Plucked | Cut | Plucked | Cut | Plucked | Cut |
| Female | Mean | 386 | 384 | 81.5 | 84.7 | 1.49 | 1.76 | 7.18 | 8.07 |
| | Max | 400 | 400 | 104.3 | 105.2 | 2.62 | 4.37 | 12.12 | 19.21 |
| | Min | 362 | 355 | 57.1 | 57.3 | 0.84 | 0.85 | 3.73 | 3.89 |
| Male | Mean | 390 | 388 | 84.1 | 89.7 | 1.51 | 1.58 (1.84) | 7.11 | 6.87 (7.96) |
| | Max | 412 | 400 | 106.4 | 113.2 | 2.91 | 3.26 (8.10) | 14.31 | 13.35 (34.29) |
| | Min | 375 | 369 | 66.0 | 66.5 | 0.90 | 0.82 | 4.04 | 3.40 |
| Total mean | | 388 | 386 | 82.8 | 87.3 | 1.50 | 1.66 | 7.14 | 7.44 |

**Table 2.** Overview of the measured results (total feather length, weight of feathers, and CORTf) of 46 Greater Flamingos (*Phoenicopterus roseus*) compared between sexes, with 26 females and 20 males, and sampling method (plucked vs. cut); 7 to 10 feathers were used for analysis.

| Sex | | Total Length of Feathers (in mm) | | Weight of Feathers (in mg) | | CORTf (in pg/mm) | | CORTf (in pg/mg) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plucked | Cut | Plucked | Cut | Plucked | Cut | Plucked | Cut |
| Female | Mean | 368 | 373 | 63.9 | 70.7 | 2.56 | 2.70 | 14.95 | 14.41 |
| | Max | 393 | 395 | 76.8 | 83.4 | 6.29 | 7.22 | 35.28 | 35.61 |
| | Min | 236 | 333 | 39.4 | 56.6 | 1.26 | 1.29 | 6.98 | 5.83 |
| Male | Mean | 371 | 372 | 77.7 | 83.7 | 2.44 | 2.54 | 11.65 | 11.25 |
| | Max | 393 | 396 | 89.5 | 95.6 | 4.11 | 4.21 | 18.46 | 17.71 |
| | Min | 330 | 341 | 63.2 | 74.0 | 1.68 | 1.45 | 8.18 | 6.73 |
| Total mean | | 370 | 372 | 69.9 | 76.4 | 2.51 | 2.63 | 13.52 | 13.04 |

## 4. Discussion

Overall, the samples were in very good condition, without any contamination, which allowed us to comply with the required minimum number of 45 individuals for both bird species. This provides a good basis for drawing meaningful conclusions from the results.

The results of this study show no relevant differences in CORTf according to the different sampling techniques, namely plucked versus cut feathers. Thus, our results confirm the findings of our previous study in captive Domestic Geese (*Anser anser domesticus*) and Mulard Ducks (*Cairina moschata* × *Anas platyrhynchos resp. Anas sterilis*) [20]. Likewise, the sex of the birds did not appear to have any influence on the CORTf level. Nevertheless, there are a few findings and aspects of the study that need to be discussed below.

In this study, as in two of our previous studies, we tried to standardize the samples as much as possible [20,27]. This was not only related to the living conditions and biological data of the sampled animal, but fundamentally related to the sampled feathers. We aimed to achieve standardization by choosing the same localization: feathers were both cut and plucked between the shoulders of each individual. Furthermore, we ensured that the morphology of the feathers to be examined was the same. Finally, the CORTf level was also standardized over the total length of the feathers. It must be added here, however, that perfect standardization of the samples can only be achieved by precise identification of the collected feathers, for example, by sampling the first and second wing feathers [20]. However, as we want to use this method in future field studies with airworthy birds, it

is important to find sample locations where feathers can be taken without affecting the airworthiness of the bird. Therefore, we think that feathers from the region between the shoulders are better suited, even if the standardization may be slightly impaired. This region has already been selected for CORTf measurements in Greater Flamingos [27], Broilers (*Gallus gallus domesticus*) [28], Red Kites (*Milvus milvus*) [19], Domestic Geese and Mulard Ducks [20].

The inter- and intra-assay CV values of the pool of both species were below the reference values, and the performance of the assay was considered acceptable. Whereby, the results of CORTf had a good reliability and repeatability.

Regarding the CORTf levels of the Mallards, one individual produced a value of 1.13 pg/mm in the plucked feather sample, whereas its cut sample resulted in 8.10 pg/mm. This result was considered an outlier because no values above 5 pg/mm were found in the whole group of ducks. In addition, the other sample of this individual did not create such high values. Such an outlier could have been caused by measurement errors, and therefore, this individual was excluded from the analysis, and the total examined number of Mallards was reduced to 46. The maximum CORTf value was higher in cut feathers, and the minimum value was higher for plucked feathers, which led to a higher range of CORTf levels with the cut sampling technique (see Table 1). In total, the cut samples produced slightly higher average values. However, these differences were marginal and therefore interpreted as negligible. Regarding the Greater Flamingos, no outlier was observed, but one individual produced high (>5 pg/mm) CORTf values with both removal techniques. It generated a level of 6.29 pg/mm with plucked feathers and 7.22 pg/mm with cut feathers. These were by far the highest maximum values in the group of flamingos, and we assume that this individual had an elevated CORT level in the plasma at the time of feather growth, which could be interpreted as some form of stress. If these values were excluded, the total average value would be decreased by 0.15 pg/mm. However, since these values were plausible, they were not removed. As described earlier, the overall CORTf levels of Mallards were lower compared to those of flamingos. The Greater Flamingos were still in their gray juvenile plumage. A larger variation of CORT levels in plasma has been described in some studies in young birds in contrast to adults [57,58]. However, the results for the flamingos clustered as expected, and the average difference between the two methods was not large at −0.13 pg/mm, even when compared to the Mallards (−0.16 pg/mm).

The CCC of both species were regarded as poor, with a worse mismatch for the Mallards (0.39 vs. 0.67). However, regarding the figures, the values clustered as desired and showed a good agreement (see Figures 1 and 2). In Figure 1 (Mallards), three individuals stood out, which had relatively high cut CORTf levels (>3 pg/mm), but plucked values that only ranged between 1 and 2 pg/mm. In the case of flamingos, the overall values were higher, but they produced a good match (see Figure 2). Nevertheless, four points were remarkable: two results with high plucked values (>4 pg/mm) and, in addition, low cut values (<3 pg/mm), a high cut CORTf level (>3 pg/mm) with a low plucked value (1–2 pg/mm) and an individual which created both very high plucked and cut CORTf levels. For these few values, the discrepancy between the two methods was increased. In summary, the poor CCC should not be viewed too critically since we had a good agreement in the comparison of the CORTf values within each individual between the methods, resulting in a basically coherent graph. The few results with the higher discrepancy were exceptions, which might had occurred because we were not comparing the identical feather (e.g., first wing feather) or because of an error in sample processing. In addition, blood quills dry at different times, even if they grow close together, which may also cause minimal differences in CORTf as a result of a slightly shifted time frame of feather growth. However, in general, regarding the whole group of birds, these differences are not significant.

For both species, Pearson's correlation coefficient was $r > 0$. A positive correlation implies that higher values of cut samples ($y$-axis) were associated with higher values of plucked ones ($x$-axis) (see Figures 1 and 2). Focusing on the BA-plot, the standard deviation of Mallards was 0.65, which was lower than the standard deviation of the flamingos. In

contrast, the average difference in CORTf between the two methods was closer to 0 in flamingos, and hence, there was a smaller difference than in ducks. However, overall, the average difference was very small. Regarding the 95% confidence interval, the results for Mallards were closer, with −1.44 to +1.12, in contrast to those for the Greater Flamingos (−1.61 to +1.36) (see Figures 3 and 4). Considering the figures, there was no increasing or decreasing trend of the data regarding the mean CORTf of the two methods and their respective average differences. In the case of the flamingos, the values tended to scatter around the center line (mean difference; Figure 4). The interval of mean values was closer in Mallards than in Greater Flamingos. There were three individuals outside the limits in the BA-plot of Mallards (see Figure 3), most likely because of the large difference of the CORTf values between the two sampling methods of one individual, as already seen in the CCC graph (see Figure 1). The graph for the Greater Flamingos was similar. There were three individuals outside the limits and, additionally, one with a very high mean value, and a small difference between the methods was observed (see Figure 4). This point represented the results of the individual with the high CORTf levels in both methods.

In summary, comparing the CORTf levels with standard deviation, the average difference, and the limits, the agreement between the two techniques, namely feather plucking and cutting, was better in the Mallards. However, concerning the CCC value, a better correlation was found for flamingos, whereas the figures (see Figures 1 and 2) showed equally good correlation in both species. Overall, the graphs of the BA-plot indicate good concordance between the two methods for both species. Additionally, it should be noted, regarding the CORTf levels, that the cut samples produced slightly higher average values. However, these differences were marginal and therefore negligible.

As described in the Results section, the Mann-Whitney U test yielded no significant difference between males and females. Considering the box plot, it appeared relatively symmetrical for the Mallards (see Figure 5); the whiskers had approximately the same length in both sexes. In male ducks, the minimum and maximum values were smaller than those in females. The median was exactly in the middle in females and deviated in males. Likewise, the box was larger in males, indicating greater dispersion in this group. In the group of Greater Flamingos, the whiskers were symmetrical, i.e., with an equal distance between minimum and maximum to the interquartile range, which correlated with 50% of the data (see Figure 6). However, the male flamingos produced smaller maximum and minimum values. The medians appeared central in both sexes. However, the box for the female flamingos was larger, indicating greater dispersion. In summary, sex did not appear to influence CORTf. In Greater Flamingos, a greater dispersion was observed in females, but in Mallards, dispersion was greater in males. In addition, in both species, females had, on average, higher maximum and minimum values. This result leaves room for interpretation. In a study with Common Redpolls, a lower plasma level of CORT was measured in males [37]. In another project, a correlation with body condition and plasma CORT levels was detected in female Snow Petrels but not in males [40]. Therefore, it may be necessary to consider including body mass as a variable in future studies with respect to body condition. In summary, if there were no influence of sex on CORTf level according to our study, there would be potential practical benefits. In some species, no clear sexual dimorphism is seen; this would normally require sex analysis via DNA. Considering the results of our study, a reliable comparison of CORTf levels without knowing the sex could be possible, with practical relevance to the finding of suitable subject groups unaffected by the distribution of sex within and to the comparison of CORTf values of individuals regardless of their sex. Furthermore, laboratories that perform DNA sexing from feathers require a freshly plucked feather, which would not be possible with our validated less-invasive method of feather cutting.

Drawing a comparison between the wild Mallards and the Mulard Ducks of a conventional poultry farm examined in our last study, the mean CORTf values were slightly higher (difference 0.25 resp. 0.11) in the Mulards in both methods (plucked: 1.75 pg/mm; cut: 1.77 pg/mm) [20]. This result could suggest that the farm birds might experience

more stress than the wild birds, although the stress experienced in the wild (e.g., regarding survival, nutrition, reproduction) should not be underestimated. In addition, the possible impact of the hunting season on the bird's stress level should not be forgotten when interpreting CORTf of wild mallards. There are risk assessment studies that have found an increase in flight initiation distance (FID) during the hunting season [59]. This should be kept in mind, especially in future studies when comparing wild birds with captive birds. In our case, the difference shown between Mallards and Mulard Ducks was not outstanding, most likely because of differences in age and duck species.

Regarding the juvenile wild flamingos in contrast to the adult zoo Greater Flamingos of a study by Reese et al., the zoo birds had a mean value of 11.46 pg/mm, with a maximum of 20.93 pg/mm and a minimum of 2.66 pg/mm [27]. In this study, only plucked feather samples were taken. Comparing them to the plucked samples of our juvenile individuals, they resulted in an 8.95 lower mean CORTf. However, it is not useful to directly compare the CORTf values of these groups due to the differences in age and living conditions. Interestingly, Reese et al. described that the CORTf results strongly varied between the zoological institutions (variance between institutions = 53.82%), whereas within the single populations, CORTf proved to be relatively consistent [27].

Due to the aspects of comparability of our results, it can be referred to as the 'STRANGE' framework by M. Webster and C. Rutz, published in Nature in 2020 [60]. The authors note how large the variation is in the animal world, indicating the importance of building a framework to enable accurate comparison. To consider all these influences when studying individuals, the acronym STRANGE was invented, short for Social background; Trappability and self-selection; Rearing history; Acclimation and habituation; Natural changes in responsiveness; Genetic make-up; and Experience [60]. Taking this into account, a direct comparison between different bird species seems difficult, as well as a comparison between adults and juveniles. Therefore, to create representative studies, it is highly important to create data bases and include as accurate as possible descriptions of living conditions, husbandries, and behavior or internal factors (e.g., age, sex). Considering these influencing factors is the only way to correctly interpret CORTf as a stress measure.

## 5. Conclusions

In summary, the differences between the two sampling methods were negligible. Consequently, cutting feathers is a possible alternative technique. It should be added that this method, although it is less invasive, could be burdening for the individual, as living birds must still be caught and held. Hence, we suggest that such projects should be linked with capture events for different purposes. Only then, we act in the sense of animal welfare and refinement. Furthermore, it should be recommended to include more potentially influencing factors, not only the sex, but also body mass.

Considering the results from this and from another study [20], feather cutting can be used as a sampling method for CORTf determination.

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**Institutional Review Board Statement:** This type of project does not fall under the legislation for the protection of animals used for scientific purposes, German Animal Welfare Act (copy file attached) in accordance with Directive 63/2010/EU. The ethical statement confirmed that the application for an animal experiment was not required, since the Mallards were shot for hunting purposes

before sampling. The sampling of Greater Flamingos was carried out in the context of the approved scientific ringing program in Andalusia, which began in 1986. It is planned and carried out by the Consejería de Agricultura, Pesca y Desarrollo Sostenible, the environmental agency responsible for the protection of wild species within the Junta de Andalucía. Knowledgeable people participate in all the planning and implementation, and all participants attend preparatory meetings for the proper handling of the birds.

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# 5 Diskussion

Das Ziel beider Studien war es zu untersuchen, ob die Federentnahmemethode (gerupft oder geschnitten) einen Einfluss auf die gemessenen CORTf-Spiegel hat. In den Ergebnissen der ersten Veröffentlichung konnte der Grundsatzbeweis, dass keine relevanten Unterschiede in den CORTf-Werten zwischen den zwei Federentnahmetechniken bestehen, dargestellt werden. Die zweite Studie bestätigte die Resultate der ersten und verifizierte gleichzeitig die Methode in der Anwendung bei Vögeln in der Wildbahn. Daher kann das alternative und weniger invasive Federschneiden in zukünftigen wissenschaftlichen Untersuchungen als Methode zur CORTf-Bestimmung angewendet werden und leistet damit einen aktiven Beitrag zur Verringerung von Tierversuchen.

## 5.1 Die Federproben

Die Probenqualität war in der ersten Studie durch vereinzelte Blut- und Kotverschmutzungen vermindert. Dieses Federmaterial wurde von der Analyse ausgeschlossen, um sicher zu gehen, dass die gemessenen CORTf-Spiegel keinen durch das in Kot und Blut enthaltene CORT veränderten Wert aufwiesen (Voit et al. 2020). Dadurch lag die Gesamtanzahl an untersuchten Exemplaren unter der statistisch berechneten Mindestzahl von 45 Tieren (40 Gänse und 37 Enten). Daraus folgend konnte die gewünschte Genauigkeit nicht aufrechterhalten werden, weshalb die Ergebnisse vorsichtiger interpretiert werden müssen. In der zweiten Studie hingegen waren die Federn von guter Qualität, wodurch die benötigte Mindestanzahl erreicht wurde (Voit et al. 2021). Da sowohl die erste Veröffentlichung mit einer verminderten Probenzahl als auch die zweite mit der statistisch erforderlichen Anzahl auf das gleiche Ergebnis (keine relevanten Unterschiede in den CORTf-Werten) kommen, ist davon auszugehen, dass die Resultate der ersten Studie trotz verminderter Genauigkeit zur Interpretation herangezogen werden können. Rückschließend ist darauf zu achten, dass zum einen eine genügend hohe Anzahl an Individuen beprobt wird, damit mögliche Ausschlüsse abgepuffert werden können, und zum anderen bei der Probenentnahme vermehrt auf Kontaminationen geachtet wird.

Ebenso wurde darauf geachtet, dass die Federn, die Entnahmestellen und -techniken so genau wie möglich standardisiert wurden. Bei den ausgewählten Federn spielte die Einheitlichkeit von Farbe und Morphologie eine große Rolle. Bei der Entnahmestelle wurde die Region zwischen den Schulterblättern festgelegt, da sich diese schon in anderen Studien als valide herausgestellt hatte (Carbajal et al. 2014;Monclús et al. 2018;Reese et al. 2020b;Haase et al. 2021). Hier ist jedoch der Aspekt zu beachten, dass nicht die komplett identische Feder

jedes einzelnen Individuums miteinander verglichen wurde, wodurch möglicherweise eine geringgradige Variation im CORTf-Wert entstanden sein könnte. Um diesen Gesichtspunkt zu vermeiden, müsste beispielsweise immer die erste und zweite Schwungfeder zur Untersuchung herangezogen werden. Es sollte jedoch eine Methode validiert werden, die praktikabel, z.B. auch bei Wildvogelpopulationen, ausgeführt werden kann, damit nicht die Flugfähigkeit des beprobten Individuums eingeschränkt wird. In Zukunft ist anzudenken mehrere Proben eines Vogels aus dieser Entnahmeregion auf CORTf zu untersuchen, um die Variation innerhalb eines Individuums herauszufinden. Zusätzlich ist die einheitliche Entnahmetechnik wichtig. Dies bezieht sich vor allem auf die Methode des Federschneidens. In beiden Studien wurde streng darauf geachtet die Federn möglichst hautnah, jedoch ohne die Haut zu verletzen, abzuschneiden. Dies ist zum einen wichtig, damit dem Tier kein Schmerz zugefügt wird, und zum anderen, dass keine Hautpartikel den CORTf-Wert verfälschen.

### 5.2 Statistische Analyse und Interpretation

Vor der Interpretation der CORTf-Spiegel ist die Validierung des ELISA zu analysieren. Hier dient die Bestimmung des Intra- und Inter- Assay-Variationskoeffizienten. Die Berechnungen in der ersten Studie ergaben bei den Gänsen 8,29 % (Intra-Assay) und 15,79 % (Inter-Assay) (Voit et al. 2020). Die Werte der Enten lagen bei 8,34 % (Intra-Assay) und 14,25 % (Inter-Assay) (Voit et al. 2020). Da der Variationskoeffizient schon in zwei Veröffentlichungen für Enten und Flamingos berechnet wurde (Voit et al. 2020;Reese et al. 2020b), wurde in der zweiten Studie ein Pool aus beiden Vogelarten zur Analyse hergestellt. Dies führte zu Ergebnissen von 9,44 % (Intra-Assay) und 11,96 % (Inter-Assay) (Voit et al. 2021). Der Referenzbereich für den Intra-Assay-Variationskoeffizienten liegt bei unter 10 % und der des Inter-Assay bei unter 15 % (Andreasson et al. 2015;Hanneman et al. 2011;Moosavi und Ghassabian 2018). Somit bestand nur bei den Gänsen eine nicht optimale Inter-Assay-Leistung. Jedoch lag dieser Wert nur geringgradig über dem Referenzbereich, wodurch insgesamt die Leistung der Assays in beiden Veröffentlichungen mit einer akzeptablen Wiederholbarkeit und Zuverlässigkeit eingestuft werden konnten.

Im nächsten Schritt wurden die CORTf-Werte verglichen. Hierbei wurden die Minimum-, Maximums- und Durchschnittswerte der beiden Federentnahmemethoden, gerupft und geschnitten, gegenüberstellend betrachtet. Zusätzlich kamen in der zweiten Studie eine Aufspaltung der Werte zwischen männlichen und weiblichen Tieren, die ebenfalls analysiert wurden. Es fielen vereinzelte Individuen auf, die bei einer der beiden Entnahmemethoden einen CORTf-Wert aufwiesen, der mehr als 5 pg/mm höher war als der der anderen Probe des

gleichen Individuums. Diese Spiegel lagen weit über den durchschnittlich gemessenen Werten und ließen sich nur durch Fehler in der Messung oder in der Laboranalyse zurückführen. Diese wurden als Ausreißer interpretiert und von der weiteren Analyse ausgeschlossen. Wohingegen Exemplare, die bei beiden Entnahmemethoden einen überdurchschnittlich hohen Wert an CORTf (> 5 pg/mm) zeigten, nicht als Ausreißer bezeichnet werden dürfen. Diese mussten differenziert betrachtet werden als Individuen, die einen erhöhten CORT-Spiegel in der Zeit des Federwachstums hatten, welcher auf Stresseinfluss zurückgeführt werden kann. Zusammenfassend sind innerhalb der untersuchten Tiergruppen keine massiven Differenzen beim CORTf-Vergleich aufgefallen. Die Unterschiede waren so marginal, dass die Federentnahmemethode keinen relevanten Einfluss auf den Hormonwert zu haben schien.

Bei der nachfolgenden statistischen Untersuchung mittels CCC und Bland-Altmann-Plot wurde derselbe Rückschluss gezogen. Nur der CCC-Wert konnte nicht eindeutig interpretiert werden. In beiden Studien lagen die Resultate unter dem gewünschten Referenzwert von 0,9 (McBride 2005). Jedoch sollten die niedrigen CCC-Ergebnisse nicht zu kritisch gesehen werden, da beim Vergleich der CORTf-Werte innerhalb jedes Individuums eine gute Übereinstimmung zwischen den Methoden herrschte, was zu einem grundsätzlich kohärenten Diagramm führte. Die wenigen Ergebnisse mit einer höheren Abweichung waren Ausnahmen, die möglicherweise darauf zurückzuführen waren, dass nicht die gleiche Feder verglichen wurde oder ein Fehler bei der Probenverarbeitung entstanden war. Zusätzlich trocknen Federkiele zu unterschiedlichen Zeiten, auch wenn sie dicht beieinander wachsen, was ebenfalls zu minimalen Unterschieden bei CORTf führen kann. Auf die gesamte Gruppe der Vögel bezogen sind diese Unterschiede jedoch nicht signifikant. Die Analyse des Bland-Altmann-Plots stellte sich in beiden Studien eindeutig dar. Hier befanden sich die Ergebnisse gestreut im 95%-Konfidenzintervall, ohne eine auf- oder absteigende Tendenz zu zeigen.

Im Mann-Whitney U Test der zweiten Studie wurde kein Einfluss des Geschlechts auf den CORTf-Spiegel festgestellt. Da in anderen Untersuchungen eine Variation im Plasma-CORT zwischen den Geschlechtern, auch in Verbindung mit dem Körpergewicht und der Konstitution, herausgefunden wurde (Angelier et al. 2009;Fairhurst et al. 2014), wäre es für künftige Projekte anzudenken, den Faktor des Körpergewichts in die Analyse mit einzubeziehen. Zusätzlich ist hier anzumerken, dass die Saison, egal ob Brutsaison oder Paarungszeit oder andere, durch ihren großen Einfluss auf den Hormonhaushalt immer eine Rolle in der Interpretation der Daten haben sollte (Cockrem 2013). Im Rückschluss darauf, dass in der Untersuchung der vorliegenden wissenschaftlichen Arbeit das Geschlecht keinen Einfluss auf die CORTf-Spiegel zu haben scheint, ergeben sich potenzielle praktische Vorteile. Wenn Vögel analysiert werden, die keinen offensichtlichen Geschlechtsdimorphismus zeigen, kann eine Geschlechtsbestimmung mittels DNA-Analyse auch aus einer Feder durchgeführt

werden. Die Labore benötigen hierzu eine frische, vollständige Feder mit vorhandenem Calamus, welche folglich gerupft werden müsste. Dies wäre mit der in dieser Arbeit validierten, weniger invasiven Entnahmemethode nicht möglich.

### 5.3 Fazit

Zusammenfassend kann gesagt werden, dass die geringen Unterschiede der CORTf-Werte in den beiden Federentnahmemethoden, geschnitten und gerupft, vernachlässigbar sind (Voit et al. 2020;Voit et al. 2021). Daraus folgend können beide Techniken zur Analyse von CORTf angewendet werden. Da jedoch marginale Unterschiede in CORTf bestehen, ist die alleinige Interpretation des Wertes nicht anzuraten, sondern diesen in Kombination mit möglichst genauen biologischen Daten und Verhaltensbeobachtungen im Zeitraum des Federwachstums zu analysieren.

Die hier validierte Methode des Federschneidens stellt im Sinne des Tierwohls eine weniger invasive Beprobung dar, da dem Tier der Schmerz des Rupfens erspart wird. Hierbei wird in beiden Veröffentlichungen von „weniger invasiv“ gesprochen, da das Fangen und Halten des Vogels an sich schon Stress für das Individuum bedeutet. Deshalb wird eine Verknüpfung der Probenentnahme mit anderen Aktionen, wie beispielsweise dem Kennzeichnen oder dem Einwintern, bei denen der Vogel ohnehin gefangen wird, empfohlen. Nur dann wird im Sinne des Tierschutzes und der Verfeinerung (Refinement) und Verringerung (Reduction) von Tierversuchen gehandelt (Directive 2010/63/EU). Durch diese alternative Entnahmemethode entstehen neue Möglichkeiten weitere Studien zum Monitoring des Wohlergehens von in Menschenobhut gehaltenen Tieren und von Wildvögeln durchzuführen.

# 6 Zusammenfassung


**Validierung und Evaluierung einer Alternativen Probenentnahmetechnik zur Corticosteronbestimmung in Federn**

Ziel der vorliegenden Arbeit ist es, eine alternative und schmerzfreie Federprobenentnahmemethode zu validieren. Zukünftig können die Federn, die zur späteren CORTf-Analyse gesammelt werden, bei lebenden Vögeln nahe der Haut abgeschnitten werden, anstatt sie zu rupfen. Hintergrund dieser Studie bildet die wissenschaftlich tierbasierte Untersuchung des Wohlbefindens von in Menschenobhut gehaltenen Vögeln mit unterschiedlichem Flugstatus. Das Studiendesign wird diesbezüglich durch eine Kombination aus der Messung und Interpretation von CORTf und Verhaltensbeobachtungen aufgebaut.

Der bisherige Standard der Probenentnahme zur CORTf-Bestimmung stellt das Federrupfen dar, das in Deutschland und anderen europäischen Ländern als Tierversuch eingestuft wird. Diese Arbeit handelt im Sinne des Tierschutzes, um die Belastung der Tiere zu vermindern, die Verfahren zu verfeinern und letztlich die Anzahl der Tierversuche zu reduzieren. Zur Bestätigung der Hypothese, dass die Entnahmemethode selbst keinen relevanten Einfluss auf gemessene CORTf-Spiegel hat, wurden von jedem zu untersuchenden Vogel sowohl gerupfte als auch geschnittene Federn gesammelt. Insgesamt sind zwei Vogelarten aus einer konventionellen Freilandhaltung, die beide die gleichen Lebensbedingungen hatten, und zwei Wildvogelarten aus unterschiedlichen Lebensräumen untersucht worden. Erstere Gruppe bildeten die Hausgänse (*Anser anser domseticus*) und die Mularden-Enten (*Anas sterilis* bzw. *Cairina moschata domestica* x *Anas platyrhynchos domesticus),* ein Hybrid aus der Moschusente (*Cairina moschata domestica*) und der Pekingente (*Anas platyrhynchos domesticus*). Die zweite Gruppe wurden durch eine Population von Großen Flamingos (*Phoenicopterus roseus*) aus Spanien und Stockenten (*Anas platyrhynchos*) aus Deutschland verkörpert.

Die CORTf-Werte wurden mithilfe eines ELISA gemessen. Die ermittelten Werte wurden in ihren Mittelwert, ihrer Standardabweichung und ihren durchschnittlichen Unterschieden verglichen. Anschließend wurden die CORTf-Werte je nach Probeentnahmeverfahren mit dem Konkordanz-Korrelationskoeffizienten (CCC) analysiert. Eine Gegenüberstellung der Unterschiede der beiden Methoden mit den jeweiligen Mittelwerten fand mit der Erstellung des Bland-Altmann-Plots statt. Das Geschlecht als möglicher Einflussfaktor auf CORTf wurde mit dem Mann-Whitney U Test bei den Großen Flamingos und Stockenten untersucht. Der CCC-Wert zeigte keine gute Übereinstimmung, wohingegen seine Grafik, der Pearsons Korrelationskoeffizient und der Bland-Altmann-Plot eindeutig waren. Die durchschnittlichen

Unterschiede zwischen den Methoden waren für alle vier Arten marginal. Zusammenfassend lässt sich sagen, dass jegliche Abweichungen oder Unterschiede zwischen den beiden Entnahmetechniken vernachlässigbar waren. Daher erscheint die alternative, weniger invasive Federprobenentnahmemethode ebenso geeignet zu sein wie die bisherige Standardmethode. Ein signifikanter Unterschied zwischen weiblichen und männlichen Tieren konnte nicht festgestellt werden. Dennoch verdeutlichen die Ergebnisse, dass eine alleinige Interpretation der CORTf-Werte nicht sinnvoll ist, sondern diese in Zusammenhang mit anderen Parametern, wie biologischen Daten und Verhaltensbeobachtungen, analysiert werden sollten. Es sind weitere Studien empfohlen, um eine größere Basis an Daten zu erschaffen.

## 7 Summary


**Validation and Evaluation of an Alternative Sampling Technique for Corticosterone Measurements in Feathers**

The aim of this work is to validate an alternative, non-painful and therefore less-invasive feather sampling method. In the future, feathers collected for subsequent CORTf analysis can be cut near the skin of living birds instead of plucking them. The background of this study is a science and animal based investigation on the welfare of birds under human care with different flight status. In this regard, the study design is built by a combination of measurement and interpretation of CORTf and behavioral observations.

The previous standard sampling method for CORTf measurements is feather plucking, which is considered as an animal experiment in Germany and other European countries. This work acts in the sense of animal welfare to reduce the number of animal experiments and refinement of procedures. To confirm the hypothesis that the sampling method itself has no effect on measured CORTf levels, both plucked and cut feathers were collected from each bird being examined. A total of two bird species from a conventional free-range poultry farming, which had the same living conditions, and two wild bird species from different habitats were studied. The former group consisted of domestic geese (*Anser anser domseticus*) and Muscovy ducks (*Anas sterilis* or *Cairina moschata domestica* x *Anas platyrhynchos domesticus*), a hybrid of Muscovy duck (*Cairina moschata domestica*) and Peking duck (*Anas platyrhynchos domesticus*). The second group were represented by a population of Greater Flamingos (*Phoenicopterus roseus*) from Spain and Mallards (*Anas platyrhynchos*) from Germany.

CORTf levels were measured using an ELISA. The values obtained have been compared for their mean, standard deviation, and average differences. Subsequently, the CORTf values were analyzed with the concordance correlation coefficient (CCC) depending on their sampling method. A comparison of the differences of the two methods with their respective mean values took place with the creation of the Bland-Altmann plot. Sex as a possible influencing factor to CORTf was examined using the Mann-Whitney U test in Greater Flamingos and Mallards. The CCC value did not show good agreement, whereas its graph, the Pearson's correlation coefficient, and the Bland-Altmann plot were acceptable. The average differences between the methods were marginal for all four species. In summary, any deviations, or differences between the two sampling techniques were negligible. Therefore, the alternative, less invasive sampling method appears to be as suitable as the previous standard method. No significant difference between females and males was found. Nevertheless, the results illustrate that a single interpretation of CORTf values is not useful, they should rather be analyzed in

combination with other parameters, such as biological data and behavioral observations. Therefore, further studies are recommended to create a larger pool of data.

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# 9 Publikationen

## 9.1 Originalartikel

Voit, Marielu, Roswitha Merle, Katrin Baumgartner, Lorenzo von Fersen, Lukas Reese, Mechthild Ladwig-Wiegard, Hermann Will, Oriol Tallo-Parra, Annaïs Carbajal, Manel Lopez-Bejar, und Christa Thöne-Reineke. 2020. "Validation of an Alternative Feather Sampling Method to Measure Corticosterone." *Animals* 10(11): 2054.

Voit, Marielu, Katrin Baumgartner, Lorenzo von Fersen, Roswitha Merle, Lukas Reese, Mechthild Wiegard, Hermann Will, Oriol Tallo-Parra, Annaïs Carbajal, Manel Lopez-Bejar, und Christa Thöne-Reineke. 2021. "Comparison of Two Different Feather Sampling Methods to Measure Corticosterone in Wild Greater Flamingos (*Phoenicopterus roseus*) and Wild Mallards (*Anas platyrhynchos*)." *Animals* 11(10): 2796.

## 9.2 Beteiligung der Autoren am Originalartikel

Marielu Voit: Beteiligung an der Entwicklung des Studienaufbaus, Vorbereitung und Studienplanung, Beteiligung an der Bereitstellung der Versuchsgruppen, Durchführung der Probenentnahme vor Ort, Beteiligung an der Laborarbeit, Auswertung der Laborergebnisse, Studiendurchführung, Visualisierung der Ergebnisse, Verfassen der Ursprungsartikel und Einfügen der Änderungen durch Koautoren und Gutachter

Koautoren: Beteiligung an der Entwicklung des Studienaufbaus, Bereitstellung von Ressourcen, Vorbereitung, Studienplanung, Konzeptplanung, Laboranalyse, Beteiligung an der Interpretation der Ergebnisse, Supervision, Review der Ursprungsartikel, Finanzierungsbeschaffung

## 9.3 Kongressbeiträge

Voit, Marielu, Roswitha Merle, Katrin Baumgartner, Lorenzo von Fersen, Lukas Reese, Mechthild Ladwig-Wiegard, Hermann Will, Oriol Tallo-Parra, Annaïs Carbajal, Manel Lopez-Bejar, und Christa Thöne-Reineke: Corticosteronuntersuchungen im Hinblick auf Tierschutzaspekte des Flugunfähigmachens von Vögeln in zoologischen Einrichtungen –

Optimierung der Probenentnahmetechnik zur Corticosteronbestimmung in Federn. DVG-Tierschutztagung, online – 23.-25.03.2022

### 9.4 Posterbeiträge

Voit, Marielu, Roswitha Merle, Katrin Baumgartner, Lorenzo von Fersen, Lukas Reese, Mechthild Ladwig-Wiegard, Hermann Will, Oriol Tallo-Parra, Annaïs Carbajal, Manel Lopez-Bejar, und Christa Thöne-Reineke: Validierung einer alternativen Federentnahmemethode zur Corticosteron-Bestimmung. 4. Jahrestagung der Fachgruppe „Zier-, Zoo- und Wildvögel, Reptilien, Amphibien und Fische (ZZWARF)“, online – 04.-06.03.2021

Voit, Marielu, Roswitha Merle, Katrin Baumgartner, Lorenzo von Fersen, Lukas Reese, Mechthild Ladwig-Wiegard, Hermann Will, Oriol Tallo-Parra, Annaïs Carbajal, Manel Lopez-Bejar, und Christa Thöne-Reineke: Validation of an Alternative Feather Sampling Method to Measure Corticosterone. UFAW Animal Welfare Conference, UK, online – 29.-30.06.2021

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## 13 Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Nürnberg, 01.07.2022 Marielu Voit


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