# NON-INVASIVE MONITORING OF STRESS IN WILD ASIAN ELEPHANTS (*Elephas maximus*) IN PENINSULAR MALAYSIA

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# Abstract

Translocation of wild Asian elephants (*Elephas maximus*) is used extensively to mitigate human-elephant conflict (HEC) in Peninsular Malaysia since 1974. Very little is known about the fate of translocated elephants after relocation due to challenges in observing elephants in the dense rainforest. Advances in wildlife endocrinology suggest that faecal glucocorticoid metabolites (fGCM) can be used to study adrenal activity remotely, to assess the Hypothalamic-Pituitary-Adrenal (HPA) axis response towards stressors.

The aim is to assess the impact of translocation on wild Asian elephants in Peninsular Malaysia using faecal endocrinology and GPS technology. The specific objectives are: (i) adapting hormone sampling methods for use under tropical field conditions, (ii) comparing fGCM concentrations between translocated and local resident elephants using enzyme immunoassay, and (iii) quantifying gastrointestinal parasite eggs and microflora ciliates in faecal samples to detect signs of immunosuppression.

We found that Asian elephant's fGCM (80 dungpiles, 685 subsamples) are stable up to eight hours in the field. From the monitoring of wild elephants at the release sites, between two months up to a year, translocated elephants (N=5) had lower fGCM concentrations in comparison to local resident elephants (N=4; Linear Mixed Models: t=-2.77, df=7.09, $P$=0.027). There were no differences in gastrointestinal parasite egg counts ($P$>0.05) or microflora ciliate counts ($P$>0.05) between translocated and local resident elephants.

In conclusion, translocation does affect elephant physiology but this is in the opposite direction from that expected – a prolonged decrease rather than increase of adrenal activity. It is unknown if these conditions could cause immunosuppression, but it could adversely affect stress response and health of the elephant (e.g. adrenal insufficiency, chronic fatigue or Post-Traumatic Stress Disorder). When assessing HEC mitigation, conservation authorities and other stakeholders need to consider that translocation may not be the best solution for HEC, as it will have long-term consequences on elephants' health.

(300 words)

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# List of Contents

# List of Tables

# List of Figures

# List of Acronyms

| | |
|---|---|
| ACTH | Adrenocorticotropic hormone |
| AIC | Akaike Information Criteria |
| ANOVA | Analysis of variance |
| BIC | Bayesian Information Criteria |
| BTFC | Belum-Temengor Forest Complex |
| CITES | Convention on International Trade in Endangered Species of Wild Fauna and Flora |
| CPG | Count per gram |
| CRH | Corticoptropin-releasing hormone |
| CNS | Central Nervous System |
| DEX | Dexamethasone |
| DWNP | Department of Wildlife and National Parks Peninsular Malaysia |
| DNA | Deoxyribonucleic acid |
| EIA | Enzyme Immunoassay |
| FAO | Food and Agriculture Organisation of the United Nations |
| LMM | Linear Mixed Model |
| GIN | Gastrointestinal nematodes |
| GPS | Global Positioning System |
| HEC | Human-elephant conflict |
| HPLC | High-performance liquid chromatography |
| HPA axis | Hypothalamic-Pituitary-Adrenal axis |
| IUCN | International Union for Conservation of Nature |
| LR | Likelihood-Ratio |
| FAO | Food and Agriculture Organisation |
| fGCM | Faecal glucocorticoid metabolites |
| MEME | Management & Ecology of Malaysian Elephants |
| MIKE | Monitoringof the Illegal Killing of Elephants |
| ML | Maximum Likelihood |
| NECC | National Elephant Conservation Center |
| PTSD | Post-Traumatic Stress Disorder |
| REML | Restricted Maximum Likelihood |
| SD | Standard Deviation |
| SE | Standard Error |
| SPE | Solid Phase Extraction |
| Th1 | T helper cells Type 1 (cellular immune system) |
| Th2 | T helper cells Type 2 (humoral immune system) |
| UK | United Kingdom |
| UNMC | University of Nottingham Malaysia Campus |
| UNUK | University of Nottingham United Kingdom |
| MNS | Malaysia Nature Society |
| VHF | Very High Frequency (radio-collar signal) |
| vs. | versus |
| WCS | Wildlife Conservation Society |
| WWF | World Wide Fund for Nature |

# 1 Introduction

## 1.1 Background

The Asian elephant (*Elephas maximus*) is the largest terrestrial animal in Asian ecosystems and a species that play significant ecological roles in dispersing seeds (Campos-Arceiz et al., 2012; Campos-Arceiz and Blake, 2011), modifying landscapes (Haynes, 2012) and altering vegetation structures (Corlett, 2007; Cristoffer and Peres, 2003). The Asian elephant is listed as "endangered", by the IUCN Red List of Threatened Species, due to population loss and reduction in geographical range in recent decades (Choudhury et al., 2008). The fragmentation of Asian elephant's habitat further aggravates human-elephant conflict (HEC), resulting in crop damage, property destruction, and loss of lives for both elephants and humans (Choudhury et al., 2008; Sukumar, 1989).

Peninsular Malaysia has seen its total forest cover shrunk from approximately 80-90% in the 1920's to just 37.7% in 2010 (Aiken, 1994; Lim and Suksuwan, 2007; Miettinen et al., 2011; MNS, 2009). The emphasis on anthropogenic development for the past few decades in Malaysia (Nagulendran et al., 2016), has led to disappearance of local elephant populations from several States on the west coast of Peninsular Malaysia (i.e. Selangor, Melaka and Perlis; FAO, 2002; Saaban et al., 2011) and the local extinction trend is spreading as more forested areas are being developed (Tan, 2016, see Figure 1-1). Like elsewhere, wild elephants in Peninsular Malaysia often crop-raid nearby plantations and settlements causing economic losses (Saaban et al., 2011), and the presences of elephants are generally not tolerated by local villagers (Ponnusamy et al., 2016). Without tolerance, it is very difficult to achieve co-

existence between people and wild elephants, even when crop damages can be reduced (Ponnusamy et al., 2016). This results in pressure on the government to resolve HEC by removing the elephants.

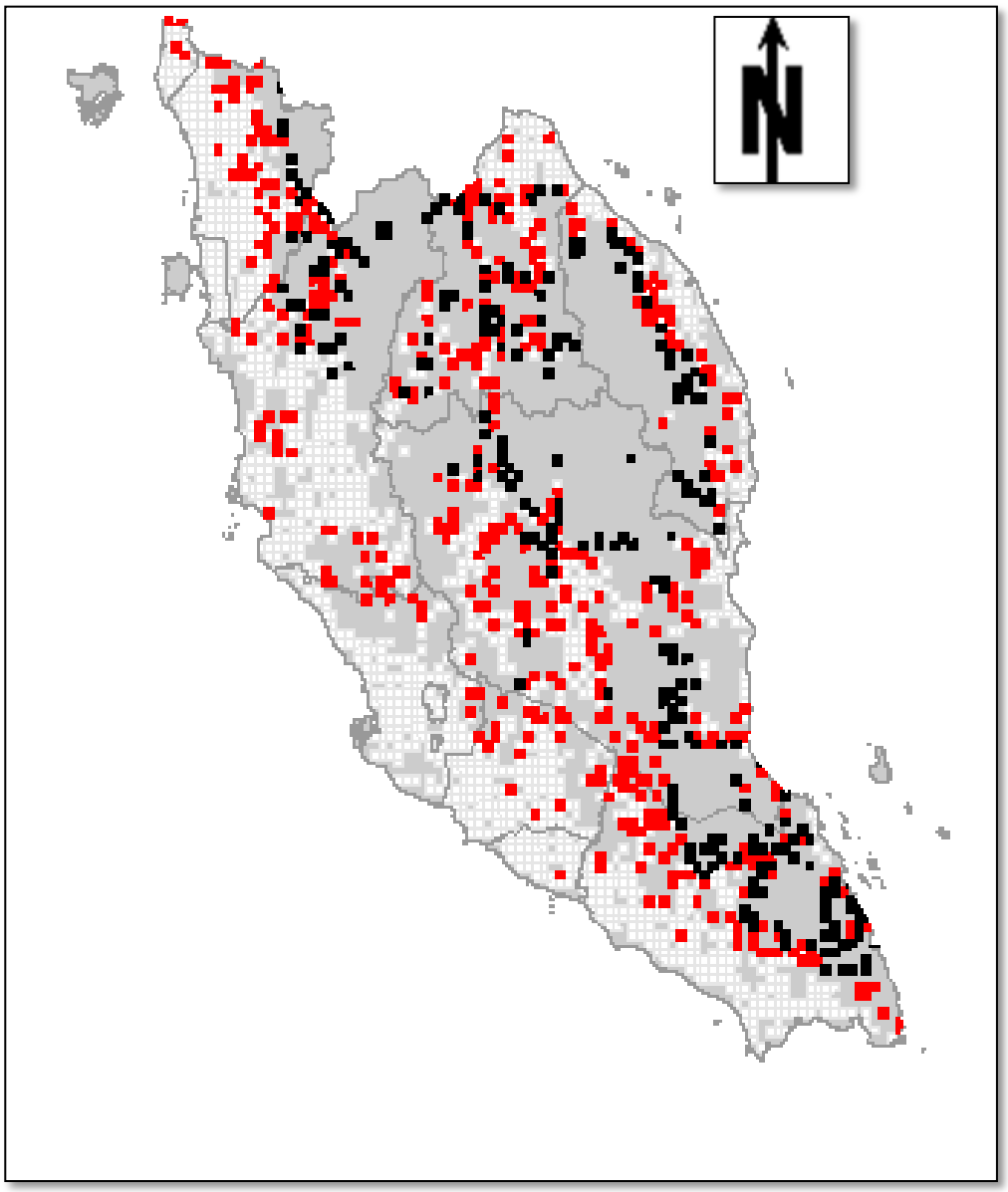


**Figure 1-1: Past and present distribution of elephants in Peninsular Malaysia in human-occupied landscape [Source:** (Tan, 2016)**, the red dots represent presences detected 40 years ago but currently locally extinct, while the black dots represent where elephants are present now]**

In Peninsular Malaysia, the responsibility of mitigating HEC falls solely on the Department of Wildlife and National Parks (DWNP), formerly known as the Games Department during the British colonial times. When Malaysia was still a young country and just started expanding its plantations, elephants involved in crop raiding were considered agriculture pests and were culled (Khan, 2014). However, in 1972, elephants' status under the Act 716 was elevated from 'protected' to 'totally protected' in Peninsular Malaysia. In subsequent years, DWNP set-up an elephant translocation unit to move elephants from conflict areas and stopped the culling of HEC elephants (Saaban et al., 2011). Between 1974 to 2011, there had been 679 wild elephants captured from HEC areas whereby 398 were relocated to Protected Areas while the rests were either retained in captivity or did not survive (FAO, 2002; Lee, 2015; Lim, 2012; Saaban et al., 2011). Considering that wild elephant population in Peninsular Malaysia was estimated to be in the range of 1223 to 1677 individuals (Saaban et al., 2011), translocation could have a large effect on the wild population.

Elephant translocation, as practiced in Peninsular Malaysia, is an intrusive procedure. Once the wild elephant is captured, it is restrained with chains for a few days until the DWNP team brings over a few captive elephants to help with transporting the wild elephant to the release site. The whole process of capture and relocation can last from a few days up a week before the elephant is released in a new area [see review by Daim (1995)]. Furthermore, the elephant is separated from its family or familiar social environment, and may face difficulty integrating with local elephants at the new location (de Silva et al., 2011; Fernando et al., 2012; Pinter-Wollman et al., 2009). Little is known about the effect of translocation on the survival and health of Asian elephants. To date, one study from Sri Lanka, utilizing GPS (Global Positioning

System) satellite collars, found that translocated elephant bulls spent a lot of effort on attempts to return to their original home range, resulting in more HEC cases and higher mortality for the animals (Fernando et al., 2012).

It is challenging to study elephants in the forest due to the thick vegetation foliage that prevents direct sighting and the elusive nature of elephants in the jungle (Blake and Hedges, 2004); this has also resulted in limited information about forest elephants' population and social structure, in comparison to African savannah elephants (Blake and Hedges, 2004; Walsh and White, 1999). In 2011, the Management & Ecology of Malaysian Elephants (MEME), a collaborative research project between the University of Nottingham Malaysia Campus and DWNP, began collaring wild elephants in Peninsular Malaysia with GPS satellite collars (Hii et al., 2016). This collaboration has provided the opportunity to track known translocated and local resident elephants in the field, via GPS collars and VHF (Very High Frequency) radio signals.

The use of GPS satellite collars to locate the elephants also enables the collection of non-invasive faeces for further endocrinology and faecal parasitology studies. 'Stress' is often identified as an unavoidable outcome in the process of capture, immobilisation and translocation of wildlife that increases the probability of mortality for the animal during the process and after release (Dickens et al., 2010; Hernandez, 2013; Hofer and East, 1993; Tarszisz et al., 2014; Teixeira et al., 2007). Considering that translocation of wild elephants is being carried out extensively in Peninsular Malaysia, it is timely to assess the impact from translocation on wild elephant's physiology, behaviour and ability to adapt to a new habitat.

## 1.2 Measurement of "stress"

The hypothalamic-pituitary-adrenal (HPA) axis evolved from a common evolutionary line and it is found even in the Pacific lamprey (*Entosphenus tridentatus*), one of the species belonging to the most ancient vertebrate lineage (Denver, 2009; Rai et al., 2014). There are similarities in HPA axis response towards stressors across different groups of vertebrates; often animal models (e.g. mice or rats) are utilised in studies on glucocorticoids (cortisol and corticosterone), physiology and human stress-related diseases including anxiety, depression and Post-Traumatic Stress Disorder (PTSD; Tamashiro et al., 2005; Yehuda and Antelman, 1993; Zoladz et al., 2012).

When faced with a dangerous situation (e.g., zebra chased by a lion; Sapolsky, 2004), within seconds to minutes, the body will release a cascade of hormones, including catecholamines and corticotropin-releasing hormone (CRH), into the blood stream. The instantaneous release of the first wave of hormones will influence physiological changes in the body, redirecting available energy resources to "fight or flight" (Sapolsky et al., 2000; Sheriff et al., 2011). The CRH, secreted by the hypothalamus, will enhance the pituitary's secretion of adrenocorticotropic hormone (ACTH), which in turn, will stimulate the adrenal glands to release glucocorticoids minutes after the stressful encounter (Chrousos, 1998; Sapolsky et al., 2000; Sheriff et al., 2011). Glucocorticoids are steroid hormones that will exert a slower physiological effect on the body (a few hours), and will act through a negative feedback loop to receptors in the brain, to reduce the production of CRH and ACTH after the stressor ends (Chrousos, 1992; Sapolsky et al., 2000).

Since Selye (1950) introduced his theory of "General Adaptation Syndrome" outlining the adrenal glands' role in secreting glucocorticoids as response to stimulants (stressors), more researchers have identified links between glucocorticoid concentrations and health (McEwen and Sapolsky, 1995; McEwen and Wingweld, 2003; Romero et al., 2009; Sapolsky et al., 2000). Although glucocorticoids are often termed as "stress hormones", they have important functions outside the stress response. For example, they are involved in the daily regulation of energy in the body, including influencing metabolism and appetite (McEwen, 2000; Sapolsky et al., 2000; Tsigos and Chrousos, 2002). In an acute stress scenario, glucocorticoids play a vital role in managing stress and assisting in recovery from stressors (Sapolsky et al., 2000), which includes mediating immune responses to prevent overshooting or autoimmunity, enhancing cardiovascular activation during stress, and maintaining the sensitivity of β-adrenergic receptors to catecholamines at vital locations in the body, including the heart (McEwen, 1998; Sapolsky et al., 2000; Silverman et al., 2005).

There is a wide-range of negative and positive stimuli, not limited to life-threatening situations, which can elicit response from the HPA axis and result in an increase of glucocorticoid concentrations. Examples of positive stress include social attraction and the smell of pheromones belonging to the opposite sex in humans (Lopez et al., 2009; van der Meij et al., 2010; Wyart et al., 2007) and winning a fight (social victory) in mice (Koolhaas et al., 1997). Meanwhile, the examples of negative stress from wildlife studies include disruption of social hierarchy (Creel et al., 2013; Sapolsky, 1992), social defeat (Koolhaas et al., 1997), stress of capture and transportation (Dembiec et al., 2004; Dickens et al., 2010), as well as disturbances

from anthropogenic activities such as tourism activity and logging (Thiel et al., 2008; Wasser and Hunt, 2005).

The HPA axis and body is capable of responding to some extend to acute stressors or stimulations. However if the timing and secretion pattern for glucocorticoids are disrupted (McEwen and Wingweld, 2003), the stress response is impaired (Dickens et al., 2009b), or the body reaches exhaustion due to chronic stress (Sapolsky, 1999; Selye, 1950), then glucocorticoids may have an adverse impact on health. Prolonged elevation of glucocorticoids lasting up to 72 hours can disrupt biological mechanism in brain cells (McEwen et al., 2015). Further effects of elevated glucocorticoids include dendritic shrinkage and loss of neuron spines in the hippocampus, and disruption of neurons' growth; which will have an adverse effect on memory, learning, and cognitive behaviour (McEwen et al., 2015; Sapolsky, 1999). Although not so well-known in comparison to the negative effects of elevated glucocorticoids, the consequences of glucocorticoid deficiency is also a health concern in humans. Low concentration of glucocorticoids is linked to acute adrenal crisis (potentially life-threatening; Lee and Ho, 2013), chronic fatigue syndrome (Edwards et al., 2011), Post-Traumatic Stress Disorder (Raison and Miller, 2003; Yehuda and Seckl, 2011), fibromyalgia and rheumatoid arthritis amongst other health problems (Cicchetti and Walker, 2001; Heim et al., 2000). Therefore, the ability to maintain an adequate concentration of basal glucocorticoids is vital for the body in managing daily activities (Busch and Hayward, 2009; Madliger and Love, 2014; Sapolsky et al., 2000), as well as in facing stressors or energy-intensive life-history stages such as migration (McEwen and Wingweld, 2003; Sapolsky et al., 2000; Wingweld and Kitaysky, 2002).

Another consideration is that "stress" experienced by mothers during pregnancy can influence their offspring's (F1) and grandchildren's (F2) stress response, physiology and health (Matthews and Phillips, 2012). This phenomenon has been documented for humans (Matthews and Phillips, 2012), animal models in laboratory (Franklin et al., 2010; Khan et al., 2016) and in prey species in the wild [e.g. snowshoe hares (*Lepus americanus*) predated by lynx and cayotes; see Sheriff et al., 2010b]. It is speculated that transgenerational influence of stress may have adaptive values in increasing the survival of offspring, but may result in poor health conditions, undesirable behaviour or increased susceptibility to mental conditions (Franklin et al., 2010; Matthews and Phillips, 2012).

## 1.3 Faecal glucocorticoid metabolites

The measurement of hormone concentrations in the blood provides a snapshot of the hormones circulating in an animal's body at that exact moment. In wildlife studies, however, collecting blood samples might require capturing and restraining the wild animal, which is often unfeasible and itself may elicit a stress response in the animal (Touma and Palme, 2005). The development of non-invasive monitoring methods such as faecal endocrinology have provided useful tools for monitoring reproduction and adrenal activity in wildlife, in a variety of terrestrial and aquatic species including primates, birds, elephants, bears, dugongs and whales (Burgess et al., 2012; Möstl and Palme, 2002; Sheriff et al., 2011; Wasser et al., 2000; Watson et al., 2013). Faecal samples are relatively easy to collect from free-living wildlife in comparison to other

options such as blood, saliva or urine. Furthermore, faecal glucocorticoid metabolites (fGCM) represent an average concentration of glucocorticoids produced by the adrenal glands over a period of time (depending on gastrointestinal transit time) (Palme, 2005; Touma and Palme, 2005), and are correlated with the measurement of biologically active "free" glucocorticoid concentrations in the blood plasma (Sheriff et al., 2010).

One important challenge for studies measuring steroid hormones in faeces is that each species can metabolize the native hormone differently (Touma and Palme, 2005; Wasser et al., 2000). Therefore, development of enzyme-immunoassay (EIA) for measuring fGCM requires both biochemical (assay design) and either a biological (natural stressor) or a physiological (ACTH injection) validation (Wasser et al., 2000; Watson et al., 2013). These validations will help to confirm that the assay is monitoring the targeted metabolites and ensure that there is minimal cross-reactivity with other (non-target) steroid hormone metabolites (Goymann, 2012; Heistermann et al., 2006; Palme, 2005).

When collecting fGCM samples in the field, issues to consider include the possible effects of the time elapsed from defecation to the moment of sampling, the impact of different environmental conditions (e.g. rain) experienced by the faecal sample and how activity from microbes in the gastrointestinal tract may affect initial hormone concentrations (Millspaugh and Washburn, 2004, 2003; Wong et al., 2016; see Chapter 2). While in the body, the secretion of glucocorticoids and stress recovery rate are influenced by the type of stressor, duration of stressor, the intensity of stressor's unpredictability, and past-experiences (Romero, 2004); and for social

animals, the stability of social hierarchy and presence of social support play a role as well (Sapolsky, 2015, 2005, 1990). These factors are important considerations to understand how translocation can be disruptive to elephants living in groups with hierarchies and strong social bonds (de Silva et al., 2011; Goldenberg et al., 2014). Some stressors (e.g. social defeat, predation) can have a prolonged effect (i.e. a few hours to a few seasons) on animal's physiology (Clinchy et al., 2011; Koolhaas et al., 1997; Sheriff et al., 2010b).

When designing fGCM studies, some possible confounding factors include, but not exclusive to, diet, individual variations, sex (male or female) and life history stages [see reviews by Goymann (2012), Millspaugh and Washburn (2004), Sheriff et al. (2011) and Touma and Palme (2005); as well as Cockrem (2013) on individual variations]. When studying endangered wildlife in their natural habitat, it is not possible to control for all factors, however, statistical mixed models can help reduce the noise by comparing changes within individuals (Galecki and Burzykowski, 2013; Zuur et al., 2009).

## 1.4 Faecal parasitology

Parasites play an important role in host population regulation, by influencing the survival of the hosts (Pedersen and Greives, 2008; Thomas et al., 2006); and some parasites, such as *Fasciola jacksoni* (elephant liver fluke) and *Bivitellobilharzia nairi* (blood fluke), have been implicated in causing disease and mortality in Asian elephants (Agatsuma et al., 2004; Caple et al., 1978; Fowler and Mikota, 2006).

Maintaining a healthy immune system that helps to keep parasites in check is a costly energy investment for the body at the expense of growth and other important life-history characteristics (Greer et al., 2005). When dealing with stressful situations, the HPA axis through glucocorticoids may modulate immune activities, perhaps to reinvest the energy for "fight or flight" response (Martin, 2009; Sapolsky, 2000; Sapolsky et al., 2000). The type of stressor may also determine if immunosuppression will take place. For example, a repeated but recoverable stressor for mice (e.g. handling and placing them in an empty room) will not trigger suppression of acquired-immunity (Hamilton, 1974; Tuli et al., 1995). However, exposure to predator (a cat) will induce immunosuppression of acquired-immunity, allowing reinfection of parasites, and the intensity of infection increases with the number of encounters with the predator (Hamilton, 1974).

Due to the non-invasive nature of this study, focus is given towards examining gastrointestinal parasites (i.e. nematodes and trematodes) eggs found in elephant dung. These eggs are recognizable under microscope down to taxonomic family level, but not species identification (Fowler and Mikota, 2006). In 2013, Hing et al. examined dung samples of wild Bornean elephants in Sabah and found presences of trematode, cestode and nematode eggs. Hing's study was considered the first parasitological survey for wild Bornean elephants, which also highlights that parasitology studies on wild elephants in Malaysia are very rare. For Asian elephants in Peninsular Malaysia, most parasite records comes from the captive population (Caple et al., 1978; Dr. Vellayan in Fowler and Mikota, 2006).

Faecal parasitology techniques have also enabled the study of ciliates, also known as microflora of the gut (Modrý et al., 2009; Profousová et al., 2011b), that play symbiotic role in the intestines by helping to digest nutrients from plants (Petrželková et al., 2012; Profousová et al., 2011a; Schmidt et al., 2005). Some ciliates have been implicated in certain diseases (French et al., 1996; Modrý et al., 2009), but there has been no records of ciliate diseases in elephants (Fowler and Mikota, 2006). Ciliates have various forms and can be distinguished under the microscope by their cilia, which they use for moving (Pomajbikova et al. 2010; Profousová et al. 2011).

The interactions between stress, the immune system, parasites and the gut microbiota are complex. For example, the immune system can act through a range of cytokines (protein hormones) to excite the HPA axis in order to release glucocorticoids (Silverman et al., 2005). Glucocorticoids, in turn feedback to the immune system by suppressing the synthesis of cytokines and may modulate the change from cellular (T helper cell Type 1) to humoral (T helper cell Type 2) immunity (Elenkov and Chrousos, 1999; Silverman et al., 2005; Silverman and Sternberg, 2012). Although humoral immunity is generally regarded as the immune response against extracellular parasites like helminths (Zhu and Paul, 2008), a balance of both cellular and humoral immunity is needed to keep parasite infections in check and over-emphasis on either one type of immune response could result in autoimmune-diseases or development of allergies (Maizels et al., 2004; Maizels and Yazdanbakhsh, 2003).

Parasitic helminths is known to be able to modify or suppress their host's humoral immune response to stay undetected (Elliott et al., 2007; Maizels et al., 2004; Maizels and Yazdanbakhsh, 2003). Through this process as well, gastrointestinal helminths

may alter gut microbiota composition which could create anti-inflammatory conditions that may turn out to be favourable for the health of the hosts (Elliott et al., 2007; Ramanan et al., 2016; Summers et al., 2005). As part of evolutionary arms race, the host may have adapted to counter physiological manipulation by parasites or the parasites may confer additional advantages to the hosts survival, and the absences of parasites may result in dysregulation of immune system or other health problems for the host (Elliott et al., 2007; Fellous and Salvaudon, 2009).

The density-dependent effect imposed by the immune system will naturally limit the fecundity, survival and establishment of parasites in the gastrointestinal tract of the host (Paterson and Viney, 2002). This weakens the correlation between worm burden and parasite egg counts especially in adult animals with matured immune system (Christensen et al., 1996; McKenna, 1981; Nansen and Roepstorff, 1999; Roepstorff et al., 1996; Stear et al., 1995). However, the precarious balance of parasites in the body can be shifted by stress. Experimental hormone treatments and parasite infection studies on domestic animals showed that an application of synthetic glucocorticoids can suppress innate and acquired-immune resistance in the host, which result in an increase of parasite burden and a higher shedding of parasite eggs (Douch et al., 1994; Greer et al., 2005; Huntley et al., 1992; Peña et al., 2004). These controlled experiments suggest that there are potential in developing faecal parasitology as a non-invasive tool for monitoring health of free-ranging wildlife (Chapman et al., 2006; Clough et al., 2010; Howells et al., 2011; Rothman et al., 2008), but more research is needed from free-ranging wildlife in the field (Hing et al., 2013).

## 1.5 Research aims

The overall aim of this study is to assess the physiological impact of translocation on wild Asian elephants in Peninsular Malaysia using non-invasive faecal glucocorticoid metabolites (fGCM) monitoring and faecal parasitology.

The specific objectives are:

- Adapting faecal endocrinology methods for tropical field conditions by determining the stability of faecal glucocorticoid metabolites (fGCM) under tropical climate conditions and developing a suitable fGCM field collection protocols.
- Comparing fGCM concentrations, as a measurement of physiological response from HPA axis, between translocated and local resident elephants using enzyme immunoassay, and
- To detect signs of immunosuppression by quantifying gastrointestinal parasite eggs and microflora ciliates in faecal samples.

## 1.6 Dissertation structure

This dissertation is structured in five chapters, starting with this Introduction chapter that provides the background for the study, a brief literature review for the main research topics, and the research objectives. Chapter Two, Three and Four are the outcomes from the research, presented in a scientific publication format. These three chapters are intended to be autonomous while sharing some common background and

other similarities with each other. The last chapter of this dissertation consists of a General Discussion, to discuss the implications from the findings and provide suggestions for future research. References and appendices are included at the end of the dissertation, after the last chapter.

## 1.7 Research permits

This study has obtained the necessary research permit from the Department of Wildlife and National Parks Peninsular Malaysia (JPHL&TN(IP): 80-4/2) and entry permits from Forestry Department Peninsular Malaysia and Perbadanan Taman Negeri Perak. This study has complied with research and ethics requirement of the Smithsonian National Zoological Park Institutional Animal Care and Use Committee (NZP-IACUC #10-32).

# 2 Stability of faecal glucocorticoid metabolites in Asian elephant's dung

## 2.1 Abstract

The use of faecal glucocorticoid metabolites (fGCM) has facilitated the development of non-invasive methods to study physiological conditions of endangered wildlife populations. One limitation of this approach is that fGCM concentrations are known to change over time and to vary according to different environmental conditions. The objective of this Chapter 2 was to perform a controlled dung decay experiment to understand the impact of time (since defecation) and two common environmental variables (exposure to water and direct sunlight) on fGCM concentration of Asian elephants (*Elephas maximus*). Eighty dung piles from ten Malaysian elephants were randomly exposed to a 2x2 combination of treatments (wet-shade, dry-shade, wet-sun and dry-sun) and repetitively subsampled from the time of defecation through to two days post-defecation (N= 685 faecal subsamples). Overall, the mean concentration of fGCM was stable in samples of up to eight hours old from defecation time, regardless of environmental treatment (water or direct sunlight); thereafter, the overall mean fGCM concentrations increased, peaking one day after defecation (31.8% higher than at defecation time), and subsequently decreased (reaching values 9.2% below defecation time on the second day). The treatment of sun exposure resulted in higher fGCM concentration compared to shade, while water exposure had no impact on fGCM concentrations compared to no water exposure. Hence, in field studies we recommend collecting dung samples less than eight hours old and recording shade

conditions (e.g. sun vs. shade) as a covariate for the subsequent interpretation of fGCM measurements. This study has helped to identify the optimal window for sampling in which we have a higher confidence in interpreting the results as being genuine.

**Keywords:** Adrenal activity; Asian elephant; *Elephas maximus*; dung decay; faecal glucocorticoid metabolites (fGCM); non-invasive monitoring.

## 2.2 Introduction

Faecal endocrinology has important applications for wildlife conservation because it facilitates the non-invasive monitoring of adrenal activity in wild animal populations (Möstl and Palme, 2002; Sheriff et al., 2011; Wasser et al., 2000; Watson et al., 2013). The concentration of faecal glucocorticoid metabolites (fGCM) is a reliable indicator of biologically active ("free") glucocorticoid metabolites circulating in an animal body over a period of time and, importantly, wildlife faeces are easier to collect than alternative biological samples such as blood, saliva, or urine (Möstl and Palme, 2002; Touma and Palme, 2005). Faecal GCM studies have been conducted to investigate a number of conservation-related questions (e.g. effects of anthropogenic disturbances on wildlife, translocation and human-wildlife conflict), for a range of wildlife species, including African (*Loxodonta* sp.; Ganswindt et al., 2010; Gobush et al., 2008; Pinter-Wollman et al., 2009; Viljoen et al., 2008) and Asian *(Elephas maximus;* Fanson et al., 2013; Laws et al., 2007; Watson et al., 2013) elephants.

Hormone metabolite concentrations in a faecal sample can vary over time (e.g. in the time elapsed between defecation and sample collection by a researcher) and this variation is mediated by environmental factors such as ambient temperature and moisture (Millspaugh and Washburn, 2004, 2003) as well as by the effects of bacterial activity (Möstl et al., 1999; Wasser et al., 1988). For example, fGCM concentration rapidly became unstable over time in faeces of captive and wild orangutans (*Pongo pygmaeus*; Muehlenbein et al., 2012) and showed fluctuations in faeces of African savanna elephants (*L. africana*; Stead, 2000). Moreover, exposure to different temperatures and humidity treatments had complex effects on fGCM concentration in faeces of white-tailed deer (*Odocoileus virginianus*; Washburn and Millspaugh, 2002), wild bears (*Ursus* spp.; Stetz et al., 2013), and jaguars (*Panthera onca*; Mesa-Cruz et al., 2014). These results highlight the importance of assessing fGCM stability in field conditions in studies using fGCM to monitor wildlife populations.

Asian elephants are the largest terrestrial animals in Southeast Asia and a species of great ecological significance (Campos-Arceiz et al., 2012; Campos-Arceiz and Blake, 2011) that have become endangered due to the rapid decline of their populations in recent decades (Choudhury et al., 2008). The conservation of Southeast Asian elephants is hampered by a poor understanding of their ecology and behaviour and the difficulty of studying them in tropical rainforests (Blake and Hedges, 2004). With the general aim of defining appropriate sampling protocols to study wild elephant populations in Southeast Asia, here we present a study to determine fGCM stability in semi-natural conditions in Peninsular Malaysia. The objectives are to determine (1) how long does fGCM concentration in Asian elephant faeces remain stable after

defecation; and (2) whether fGCM stability is affected by exposure to sunlight and/or water. To address these questions, we conducted a dung decay experiment with elephants in semi-natural habitats in Peninsular Malaysia.

## 2.3 Materials and Methods

### 2.3.1 Animal subjects

Faecal samples were collected from ten elephants: eight females (seven adults and one sub-adult) and two males (both sub-adults), from Kuala Gandah National Elephant Conservation Centre (NECC), in Pahang, Peninsular Malaysia. All the elephants were housed in the same paddock area at night, under similar environmental conditions. The elephants were fed a consistent diet of grasses supplemented with papaya and sugarcane; during the day they were also allowed to graze on different patches of grasslands. At noon, elephants were given a daily bath in a river. This study complied with research and ethic regulations imposed by the Malaysian government (permit: JPHL&TN(IP): 80-4/2).

### 2.3.2 Faecal sample collection

The dung piles (N = 80, three to nine boli each) were collected from the ten study individuals for five consecutive days (13 - 17 January 2013) between 4:00 and 9:00 am. Following defecation by any of the elephants, the dung piles were collected and sampled immediately (hereafter known as ‘Time 0’ samples) before being randomly

assigned to one of the 2x2 treatment combinations [wet - shade (n = 21 faecal piles), wet - sun (n = 20), dry - shade (n = 17), and dry - sun (n = 22)]. For 'wet' samples, one litre of purified water was poured over the dung pile just after the 'Time 0' sample was taken; for 'dry' samples, no water was added; for 'shade' samples, the dung piles were placed in an area under tree canopy; for 'sun' samples, the dung piles were placed in an open field. At night (7 pm to 4 am) and during two brief raining episodes, we covered all the samples with plastic sheets (placed individually over each of the dung pile) to avoid rainfall affecting the experimental treatments. During the experiment, the nearest weather station (Felda PPP Tun Razak, 03° 50'N, 102° 34'E) recorded daily averages of 6.4±2.5 h of sunlight, 25.5°C of temperature, 4±8 mm of rainfall, and 80±2% of relative humidity.

To collect faecal subsamples (N = 685 from the 80 dung piles) for hormone analysis, we used scissors to cut three small openings in different parts of the dung pile (usually on different boli) and removed faecal matter from the centre of the bolus using forceps; we then placed the sample in a ziplock bag, mixed thoroughly, and kept it frozen at -20°C until laboratory analysis. Each dung pile was coded with a unique ID number. Hormone sample bags were labelled with dung pile ID, date, and time of sampling. Faecal subsamples were taken at a range of times with sampling occurring more intensively within the first half of the day of defecation to capture initial changes in fGCM and less frequently afterwards: Time 0 (fresh defecation), 0 to 2 hours after defecation, 2 to 4 hours, 4 to 6 hours, 6 to 8 hours, 8 to 11 hours, 11 to 16 hours, 1 day, 1.5 days, and 2 days after defecation. Dung piles collected at the start of day (e.g. at 4 am) were sub-sampled more frequently than dung piles collected later in the day (e.g. at 9 am), hence the number of subsamples differs between dung piles.

### 2.3.3 Hormone analysis in the laboratory

The faecal subsamples were extracted using a wet-weight extraction technique adapted from Walker et al. (2002) and described elsewhere (Edwards et al., 2014; Watson et al., 2013), whereby 0.5 g of faecal matter was extracted with 5 ml of 90% methanol, placed on an orbital shaker overnight, dried and reconstituted in 1 ml of 100% methanol, and stored at -20°C until being analyzed with a corticosterone EIA (CJM006 supplied by Coralie Munro, UC Davis). The assay has been biologically and biochemically validated for use in Asian elephants (Watson et al., 2013). The corticosterone antiserum CJM006 cross-reactivities are published elsewhere (Watson et al., 2013) and only data with intra-assay coefficient of variation (CoV) of less than 10% and inter-assay CoV less than 15% were accepted and used for statistical analysis.

### 2.3.4 Data analysis

The fGCM concentrations were log10 transformed and analysed using cross-classified Linear Mixed Models (LMMs) to (1) determine relevant random effects and (2) assess the effect of the fixed factor treatments. One dung pile was removed from the dataset and subsequent analyses because it had been sampled less than four times during the study and three other dung piles were removed because they were considered to contain some extreme outliers (fGCM concentration > 80 ng/g). The removal of these dung piles, did not affect the outcome of the final model selection. The final dataset, therefore, included data from 76 faecal piles with a total of 660 subsamples. The fixed

factors of interest were (i) time since defecation, as a categorical variable (Time 0 (fresh defecation), 0 to 2 hours after defecation, 2 to 4 hours, 4 to 6 hours, 6 to 8 hours, 8 to 11 hours, 11 to 16 hours, 1 day, 1.5 days, and 2 days), (ii) water (wet vs. dry), (iii) shade (shade vs. sun), and (iv) the interaction between water and shade. The random factors considered were (i) variation within elephant subjects (N = 10), (ii) variation within dung piles (N = 76) and (iii) variation within days of collection (N = 5). The variation within dung piles was considered to be nested within the variation of individual elephants.

We used time as a categorical variable instead of continuous to make it easier to detect at which point in time fGCM becomes different in comparison to Time 0. To test the effect of time since defecation in fGCM concentration, the contrast is set to compare all time levels against Time 0 values.

All LMM models were fitted using the function lmer in the package lme4 (Bates et al., 2014) in R statistical environment (version 3.1.1; R Core Team, 2014). The optimal model was determined by dropping of the variables in the model one by one and comparing its significance to the model using Likelihood Ratio (LR) tests (Galecki and Burzykowski, 2013; Zuur et al., 2009). First, to choose the random covariates to retain in the final model a Restricted Estimated Maximum Likelihood (REML) and combination of LR tests, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) was used (Zuur et al., 2009). The effect of the fixed factors were assessed on the model using Maximum Likelihood by dropping the fixed factors one at a time and comparing the new model with the previous one.

Again, we kept the fixed factors that were significant for the model based on LR tests and information from AIC and BIC.

We used a supporting R package, lmerTest (Kuznetsova et al., 2015) with Satterthwaite approximation to estimate degrees of freedom to calculate *P*-values and confidence intervals for the model parameters. Density plots from simulated models were obtained (repeated 1000 values) which plotted the effect of fixed factors and random effects in the model for comparison (Galecki and Burzykowski, 2013; Orelien and Edwards, 2008). Finally, we checked the model's assumption of homogeneity and normality of residuals using Pearson standardized residuals.

## 2.4 Results

The overall mean concentration of fGCM at Time 0 (fresh defecation) was 17.35 ng/g ± 6.23 (SD) and it remained stable up to eight hours after defecation, with fluctuations within ± 6.0% from the Time 0 mean (Figure 2-1). After eight hours, the mean fGCM concentration increased considerably, being 13.20% higher at 8 to 11 h after defecation, 26.17% higher at 11 to 16 hours, and reaching its peak at 31.82% higher than Time 0 values one day after defecation. Afterwards, the fGCM concentration decreased, showing values slightly higher (14.52%) than Time 0 after 1.5 days and dropping below Time 0 values (-9.16%; 15.76±5.93 ng/g (SD)) two days after defecation.

The analysis of random effects showed that individual dung piles ($\chi^2 = 255.4$, adjusted $P < 0.001$) and individual elephants ($\chi^2 = 9.27$, adjusted $P < 0.001$) were influential effects. The covariate 'day of collection' was not influential on the data and was dropped from the model ($\chi^2 = 0.00$, adjusted $P = 0.50$). The analysis for the fixed factors, time ($\chi^2 = 183.82$, $P < 0.001$) and shade (shade vs. sun) had significant effects ($\chi^2 = 7.12$, $P = 0.008$), with sun exposure resulting in higher fGCM concentration compared to shade exposure (Table 2-1). The treatment of water did not have a significant effect on fCGM concentrations ($\chi^2 = 0.18$, $P = 0.67$). Additionally, the interactions between (1) water, shade, and time; (2) water and time; (3) shade and time; and (4) water and shade treatments had no impact on fGCM concentration ($P > 0.05$). The final LMM found time categories below eight hours were not significant ($P \geq 0.54$) while categories above 8 hours were significantly different than Time 0 ($P < 0.001$; Table 2-1). The results of the final model were confirmed by the model simulation (Figure 2-2), which demonstrated that fGCM concentration values remain stable up to eight hours after defecation but not afterwards. The model simulation also supported the retention of elephant and dung pile (nested within elephant) as random effects, and shade as a significant fixed factor.

**Table 2-1: Results from final model with *P*-values for the fixed factors and confidence intervals for intercepts and slope (beta coefficient ± standard error) generated from lme4 and lmerTest package. The parameters for random factors were obtained through simulation (x1000) by comparing the final model with both random factors and null model without random factors.**

| Type | Factor | Coefficent (S.E.) | df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|
| Fixed | Intercept (0h & sun) | 1.244 (0.026) | 17.7 | 47.710 | 1.193 – 1.297 | <0.001 |
| | Shade vs. sun | -0.039 (0.022) | 68.0 | -2.73 | -0.106 – -0.017 | 0.008 |
| | < 2h vs. 0h | -0.061 (0.014) | 580.4 | -0.21 | -0.031 – 0.025 | 0.830 |
| | 2-4h vs.0h | -0.003 (0.015) | 577.9 | -0.62 | -0.040 – 0.021 | 0.540 |
| | 4-6h vs.0h | -0.009 (0.016) | 577.7 | -0.17 | -0.033 – 0.028 | 0.860 |
| | 6-8h vs.0h | -0.002 (0.016) | 579.6 | 0.01 | -0.030 – 0.031 | 0.990 |
| | 8-11h vs.0h | 0.000 (0.016) | 577.4 | 3.75 | 0.028 – 0.088 | <0.001 |
| | 11-16h vs.0h | 0.056 (0.016) | 579.7 | 6.15 | 0.067 – 0.130 | <0.001 |
| | 1 day vs.0h | 0.099 (0.015) | 575.4 | 8.38 | 0.093 – 0.150 | <0.001 |
| | 1.5 days vs.0h | 0.122 (0.015) | 576.2 | 4.37 | 0.036 – 0.095 | <0.001 |
| | 2.0 days vs.0h | 0.066 (0.015) | 575.7 | -2.65 | -0.068 – 0.010 | 0.008 |
| Random | Elephant | 0.056 | | | 0.009 – 0.093 | <0.001 |
| | Dung pile | 0.089 | | | 0.071 – 0.109 | |
| | Random effect residual (sigma) | 0.086 | | | 0.084-0.094 | |

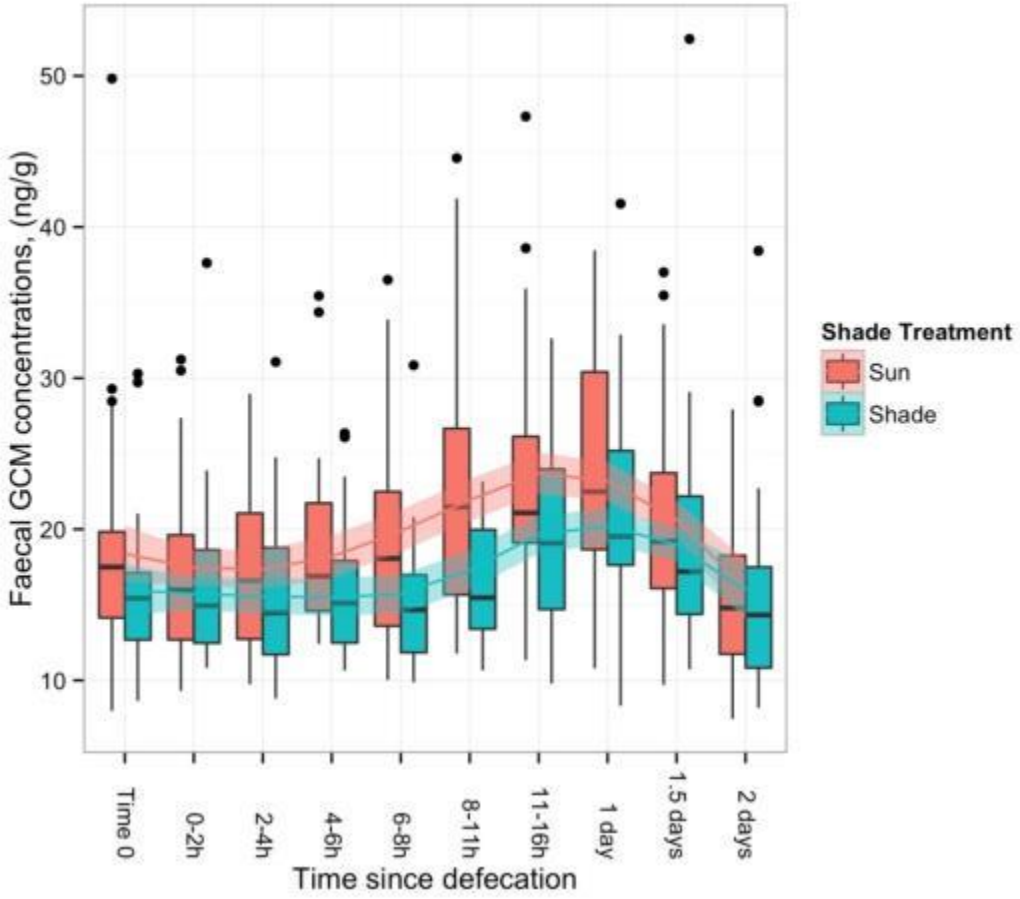


**Figure 2-1: Changes in mean fGCM concentration in Asian elephant dung (N =76 dung piles, N= 660 faecal subsamples) from time of defecation (Time 0) to 2 days post defecation. Samples under direct sun exposure (red fill) showed higher fGCM concentration compared with samples under shade (green fill).**

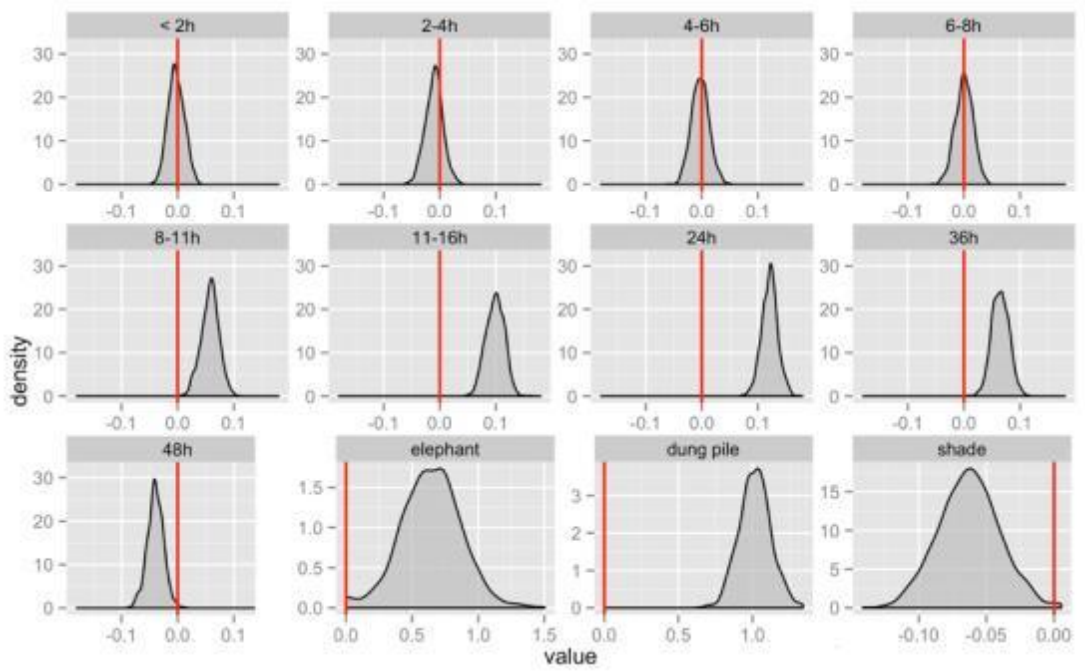


**Figure 2-2: Density plots from simulated model data (N=1000) were generated to estimate *P* values for fixed and random effects. The value of fixed effect coefficient "beta" for each time sector compared to categorical Time 0 (i.e. fresh defecation) and shade result's is the difference of shade in comparison to 'sun exposure'. If the beta value is centred around 0 (red vertical line) then there little difference between the two categories, if the value is far from 0, then the difference between the two categories is large. In terms of changes of fGCM over time, this figure shows that fGCM concentration remains stable up to 8 hours since defecation, but afterwards there are large differences with the Time 0 baseline.**

## 2.5 Discussion

We found that fGCM concentration in Asian elephants' faeces were stable for up to eight hours in semi-natural conditions in the rainforests of Southeast Asia, hence allowing us to design a reliable protocol for dung sampling for the monitoring of adrenal activity in wild elephants. This is especially relevant when tracking wild elephants with GPS satellite collars in the thick forest. In the event that the observer could not determine the exact moment when the elephant defecates, the age of the dung pile can be estimated conservatively based on when the elephant was in a particular location.

The large sample size from this study provides confidence in the results regarding the changes in fGCMs that occurred in elephant faeces up to two days old. It is important to note that fGCM concentrations became elevated after eight hours, and more rapidly when exposed to direct sunlight. The previous performance (biological validation) of this assay on captive Asian elephants suggests that fGCM concentrations above 25 ng/g reflects elevated glucocorticoid concentrations associated with a significant challenge (Watson et al., 2013). If the effect of time elapsed since defecation is ignored in a field study, researchers might erroneously conclude that a particular animal has elevated adrenal activity when it is actually an artefact of the sample's age, or of exposure to environmental conditions. Future field studies should therefore consider dung decay in their study design to determine the suitable window for sample collection.

The biological mechanisms that influence the changes in fGCMs over time could be caused by bacterial activity or transformation and breakdown of the glucocorticoid's molecular structure, resulting in differences in affinity with the antibody in the assay (Palme, 2005). Steroid hormones in the environment can be removed from the environment through sorption (absorption and adsorption), photolytic degradation (chemical decomposition under sunlight) or through microbial activity (Moschet and Hollender, 2009). For example, in a loam soil sample mixed with 80% sterilized soil, the degradation of 17β-estradiol to estrone was much slower than in fully unsterilized soil, implying that soil microorganisms play an important role in the degradation of steroid hormones (Snow et al., 2009; Xuan et al., 2008).

It is not clear why water exposure resulted in an elevation in fGCM concentrations in some studies (Mesa-Cruz et al., 2014; Washburn and Millspaugh, 2002) but not in this one. Some possible reasons are that (i) water causes changes in metabolite immunoactivity that can be picked up by some enzyme or radioimmunoassays but not by others (Mesa-Cruz et al., 2014); (ii) there could be differences in faecal forms (e.g. elephant dung is voluminous and highly fibrous, bird faeces are in the form of powder, and deer faeces are small dense pellets) or other unmeasured variables that may be influencing the interaction between water and the metabolism of the glucocorticoids present in the faeces; (iii) the high humidity in the tropical environment may also affect samples not treated with water and thus confound the effect of the water treatment, and (iv) it is possible that the amount of water utilized in the experiment was insufficient to affect fGCM concentrations. As elephant dung is full of fiber, it contains natural moisture and it has a protective mucus on the outer layer, so the addition of water may not have a major influence on the fGCMs in the

center of the boli, from where the samples were taken. In this study, we used purified water as a proxy for rainwater. We acknowledge that there are chemical differences between both and recommend future studies to use actual forest rainwater to investigate the effect of rain on fGCM stability.

Although we conducted this experiment with captive elephants—whose diet and daily activities differ from those of wild individuals—we think that the results are relevant for studies on wild elephants. Similar studies would be impossible to carry out using dung from wild elephants in the tropical rainforests of Southeast Asia because in these environments (1) elephants occur at low densities, (2) direct observation is unfeasible, and (3) getting near elephants to collect freshly-defecated dung would involve a high physical risk for the researchers. The NECC has the biggest captive elephant population in Peninsular Malaysia, and we were able to have access to eight females (65 dung piles) and two male elephants (15 dung piles) in this study. Although this sample size is adequate to study fluctuations in fGCM in individual dung piles over time, future studies could look into individual variability in fGCM in relation to "stress treatments" (see contextual interpretation of fGCM; Madliger and Love, 2014) and try to sample from a larger population with equal representation from different age groups and sex. Overall, the results have important implications for studies of free-ranging wildlife, highlighting that the time since defecation and the effect of environmental factors affect fGCM concentrations and hence they should be important considerations in field fGCM sampling protocol design.

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# 3 Comparison of faecal glucocorticoid metabolites between translocated and local wild Asian elephants.

## 3.1 Abstract

Translocation is one of the most common forms of mitigation for the conflict between people and elephants (*Elephas maximus*) in Malaysia and other Asian countries. Little is known, however, about the impact of translocation on elephants. Here we present a study that combines GPS-tracking and faecal endocrinology to monitor the physiological response of wild elephants to translocation. We tracked five translocated elephants (41 dung piles) and compared their adrenal activity with four local resident (50 dung piles) elephants at release sites in Peninsular Malaysia. We did not find prolonged elevation in faecal glucocorticoid metabolites (fGCM) in the translocated elephants; instead, between two months and a year after they were released in a new location, the translocated elephants had lower fGCM concentrations in comparison to local resident elephants (Linear Mixed Models: t=-2.77, df=7.09, P=0.027). This suggests that translocation has an effect on the animals' physiology, causing a downward shift in the production of glucocorticoids in the year following translocation. The low fGCM status may not be permanent, as two translocated elephants showed an increase in their fGCM concentration after a year from their release to a range similar to local elephants. In conclusion, translocation clearly has an effect on elephants' physiology but this is in the opposite direction as expected – a prolonged decrease rather than increase of adrenal activity. The duration of this

response and its potential adverse consequences for elephants' health and their capacity to adapt to new environment remain unclear and deserve further attention.

**Keywords:** Asian elephant (*Elephas maximus)*; faecal glucocorticoid metabolites (fGCM); non-invasive monitoring; adrenal activity; translocation, wild elephants

## 3.2 Introduction

As part of human-elephant conflict (HEC) mitigation efforts in Peninsular Malaysia, the Department of Wildlife and National Parks (DWNP) has been translocating wild elephants from conflict areas to large contiguous forest (FAO, 2002; Lim, 2012). Between 1974 and 2011, an estimated 679 elephants have been removed from conflict areas, 398 elephants were relocated, while the rest were either held in captivity or did not survive (Lee, 2015; Saaban et al., 2011).

Translocation is used as a conservation tool to repopulate wildlife in an area, or as a mitigation measure for human-wildlife conflicts (Chiarello et al., 2004; Daim, 1995; Griffith et al., 1989; Hellmann, 2013). It requires the capturing, transporting and releasing of the animal to a new location (Dickens et al., 2010; Field et al., 2007; Massei et al., 2010). The process can be disruptive, as the animal is removed from its original home range and it is not able to exert any influence on the process (Dickens et al., 2010; Rich and Romero, 2005; Teixeira et al., 2007). Findings from previous studies suggest that translocated elephants will experience challenges and threats after their release; either in finding their way back to their former range and reconnecting

with their previous social group, or in adapting to their new environment, integrating with local elephants and establishing a new home-range (Choudhury, 1993; Fernando et al., 2012; Pinter-Wollman et al., 2009). However, in comparison to other landscape, it is difficult to study elephants in the rainforest (Blake and Hedges, 2004), and not much is known about the fate of translocated elephants in Malaysia after release in their new habitat.

The development of satellite GPS collars and use of faecal endocrinology provides an opportunity to track and monitor physiological responses of individual elephants or family groups (Romero 2004; Pinter-Wollman et al. 2009; Jachowski et al. 2013; Viljoen et al. 2008). Glucocorticoids (cortisol and corticosterone), released by the hypothalamic-pituitary-adrenal (HPA) axis, is important for modulating stress response and in daily regulation of energy for the body (Sapolsky et al., 2000). Glucocorticoids are metabolised and excreted as faecal glucocorticoid metabolites (fGCM) in dung. Since an Asian elephant defecates at an average rate of 18 times a day (Hedges et al., 2005), they provide plenty of material for faecal endocrinology sampling. The challenge, however, is in collecting the dung and freezing the samples within eight hours of defecation (see Chapter 2), as the molecular structure of steroid hormone metabolites can be modified by various processes including bacterial activity, photolytic degradation, and environment sorption (Mesa-Cruz et al., 2014; Palme, 2005; Snow et al., 2009; Xuan et al., 2008). .

Past field studies of fGCM in African savannah elephants (*Loxodonta africana*) found that poaching, translocation, break down of the female social unit (loss of older matriarchs), and injury all resulted in an elevation of fGCM concentrations

(Ganswindt et al. 2010; Gobush et al. 2008; Viljoen et al. 2008; Millspaugh et al. 2007; Jachowski et al. 2013). For captive Asian elephants, it has been found that paddock renovation work and translocation between zoos caused an elevation of fGCM, implying a stress response to these situations (Fanson et al., 2013; Laws et al., 2007; Watson et al., 2013). These studies suggest that fGCM is appropriate for monitoring the effect of translocation of elephants and anthropogenic disturbances in the habitat.

### 3.2.1 Defining "stress"

The definition of 'stress' and 'stressors' can differ in terminology between researchers, depending if it is viewed as (i) a stimulation to the hypothalamic-pituitary-adrenal axis (HPA) resulting in a cascade of hormones that cause physiological changes in the body (Mason, 1971; McEwen and Sapolsky, 1995; Selye, 1950), (ii) a selective force that reduces the fitness of individuals in the population (Boonstra and Boag, 1992; Wingweld and Kitaysky, 2002) or (iii) the maladaptation of the individual's behaviour to environmental perturbation or its social community (Creel et al., 2013; Koolhaas et al., 1999; Mason, 1971; Sapolsky, 2005; Wingweld and Kitaysky, 2002). As it is not easy to study fitness and behaviour of long-living, free-ranging and elusive animals like the forest elephants, for the purpose of this study, we used glucocorticoids and their metabolites to measure the ability of the HPA axis in wild Asian elephants to response towards translocation, and their adaptation to a new environment.

The general consensus is that concentration of glucocorticoids over time which are too high (over-stimulation) or too low (under-stimulation) can be bad for health, while the middle range represents the optimal level for physiological function; this pattern can be described as an inverted-"U" relationship (Busch and Hayward, 2009; Mateo, 2008; McEwen et al., 2015; Park et al., 2006; Sapolsky, 2015). The positive manageable stress at the top of the curve can shift to either end of the spectrum depending on stressor's intensity (overstimulation or understimulation; Sapolsky, 2015), duration of the stressor (reaction, resistance or exhaustion; Selye, 1950), as well as the environment conditions (safe or unpredictable), amongst other considerations (Busch and Hayward, 2009; Romero, 2004).

There are individual differences in basal glucocorticoid concentrations and in adrenal response to stressors (Cockrem, 2013; Cockrem et al., 2009; Sapolsky, 1990). Researchers are trying to link these individual differences to theories of allostasis, and allostatic load models (Korte et al., 2007; McEwen and Wingweld, 2010, 2003) or the reactive-scope model (Romero et al., 2009), to explain the linkages between stress and a spectrum of physiological and psychological conditions and diseases. These models include a variety of parameters to measure the mediators of the HPA axis (eg. glucocorticoids, catecholamines) and linked them to physiological functions such as immune function, cardiovascular performance, behaviour and central nervous system (Korte et al., 2007; McEwen and Seeman, 1999; Romero et al., 2009). These models agree that health complications can result from either too little or too much stimulation (McEwen, 1998; McEwen and Seeman, 1999; McEwen and Wingweld, 2003; Romero et al., 2009; Sapolsky, 2015).

### 3.2.2 Translocation as chronic stress

It has been suggested that translocation will cause chronic stress and result in a 'blunted' glucocorticoids response (Dickens et al., 2010). This means, when encountering an acute stressor during a period of chronic stress, the peak concentration of glucocorticoids secreted by the adrenal glands will be lower in comparison to presences of an acute stressor only (Busch and Hayward, 2009; Dickens et al., 2010; Rich and Romero, 2005). Although the response is lower, the overall amount of glucocorticoids produced over time will be higher either due to repeated activation of the HPA axis (McEwen and Wingweld, 2003; Sapolsky et al., 2000), or through impairment of the negative feedback mechanism (Dickens et al., 2010; Romero, 2004). Based on these studies, we would expect that fGCM concentrations (being an integrated measurement of glucocorticoids over a certain period of time) would go up during translocation, and decline after the elephants are released. The reported duration of elevated fGCM seen after transportation ranged between four to eight weeks in African savanna elephants (captive-tourism Millspaugh et al., 2007; wild-translocation Viljoen et al., 2008), and up to 28 weeks in captive Asian elephants (Fanson et al., 2013). However, there is no available information on wild Asian elephants. Furthermore, there has been no consensus on chronic stress profile for wildlife due to limited data from the field (Dickens and Romero, 2013).

The overall aim of this research was to assess the impact of translocation on wild Asian elephants in Peninsular Malaysia, as this may have wider implications for the health of the population (translocated individuals, female elephants and their

offspring). The specific objective was to monitor adrenal activity in translocated elephants, and compare this to adrenal activity in local resident elephants, using faecal endocrinology. To the best of my knowledge, there has not been a longitudinal study on fGCM in wild Asian elephants that compares translocated elephants with local resident elephants in natural habitat. In addition, this study provides insight into adrenal activity for a small group of translocated Asian elephants, and how the response of these individuals fits into current knowledge of wildlife translocation and HPA response (Dickens et al., 2009a, 2009b).

## 3.3 Materials and Methods

### 3.3.1 Study Area

The study area for this project included the two largest forest complexes, Belum-Temengor Forest Complex and Kenyir Forest Complex in northern Peninsular Malaysia (see Figure 3-1). Both landscapes are important habitats for wild elephants and other wildlife including the Malayan tiger (*Panthera tigris jacksoni*), gaur (*Bos gaurus*), Malayan tapir (*Tapirus indicus*), hornbills and many others (Clements et al., 2010; Hedges et al., 2015; Rayan and Linkie, 2015).

The first study site, the Belum-Temengor Forest Complex is located in the north-west of Peninsular Malaysia, and comprised of hill dipterocarp and upper dipterocarp forest. It covers the Royal Belum State Park (1,175 km$^2$), Temengor Forest Reserve (1,489 km$^2$), state land (131 km$^2$), indigenous villages, plantations, lake, rivers and

dams and this landscape is bisected by the Gerik-Jeli East-West highway about 121 km in length (Malaysian Nature Society 2009; Clements et al. 2010; Rayan & Linkie 2015).

The second study site, Kenyir Forest Complex, is situated at the north-east of Peninsular Malaysia. It covers Taman Negara National Park (4,343 km$^2$), forest reserves (lowland-hill dipterocarp forest) with selective logging and clear-felling, lake, rivers, dams, and plantations, and a 60km road (Clements, 2013; Hedges et al., 2015).

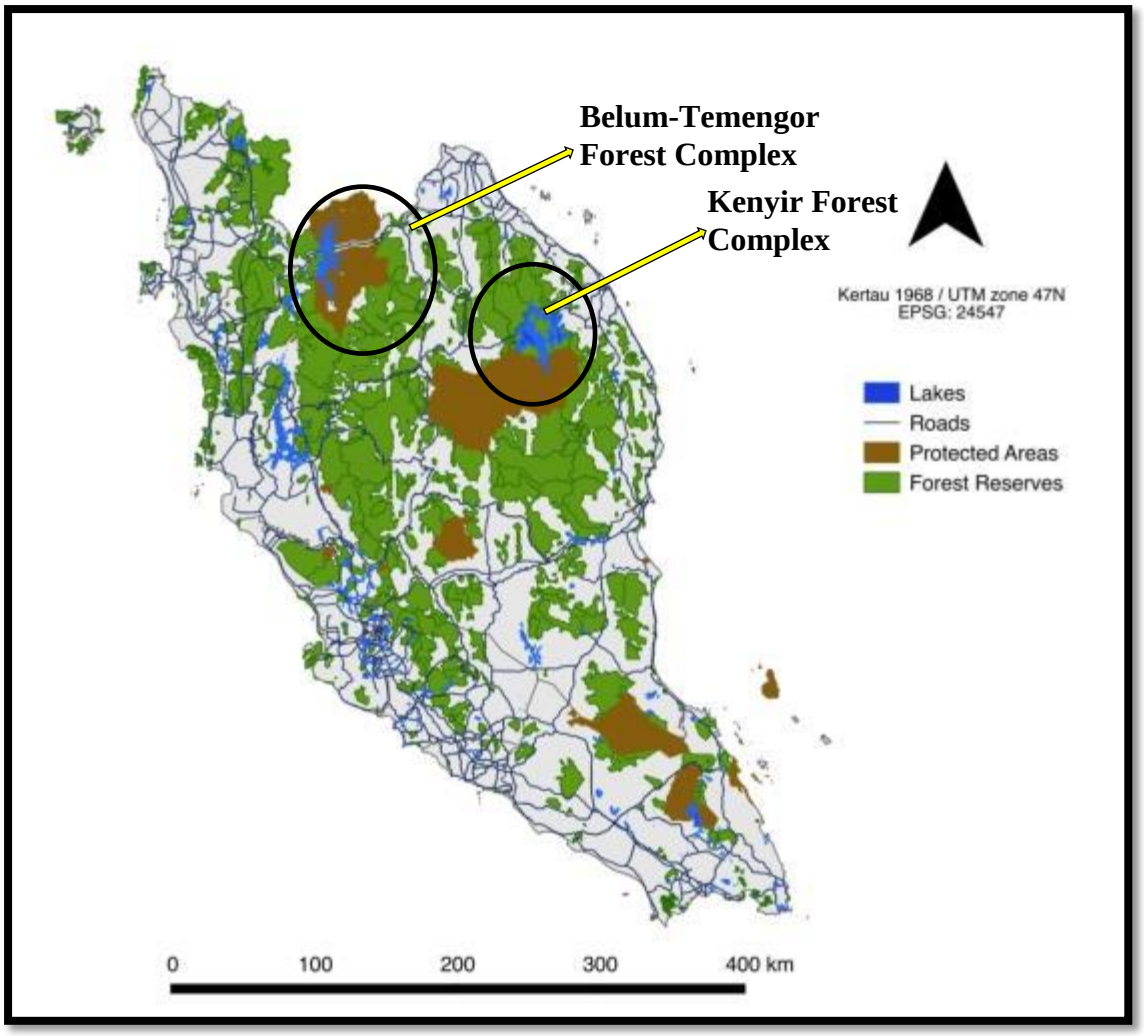


**Figure 3-1: Map showing the two study sites (Belum-Temengor Forest Complex and Kenyir Forest Complex) in Peninsular Malaysia.**

### 3.3.2 Translocation procedure and collaring

The process of capturing and translocation of wild elephants is managed by the Department of Wildlife and National Parks (DWNP). The standard operating procedure for translocation has been described in Daim (1995). In brief, the translocation process for an elephant may take from three days to a week depending on the difficulty of the landscape. When the elephant to be translocated is located, the DWNP sedates the elephant with *Immobilon*, using a dart, shot from a rifle. The

dosage for *Immobilon* is adjusted accordingly to the size of the elephant, and the estimation is based on the circumference of the front footprint of the targeted elephant (Daim, 1995; Khan, 2014). Then, the officers secure the elephant's legs with chains to a tree, before giving the elephant the revival drug, Revivon *(Diprenorphine hydrochloride).* On the day of translocation, the captured elephant is loaded on the transportation truck with the help of two trained captive elephants (*gajah denak*) belonging to the DWNP. The drug *Xylazine (Rompun)* is used for sedating the captured elephant during the process; if needed, the reversal agent *Yohimbine hydrochloride* is used for reviving the elephant during transportation (Daim, 1995; pers. comm. Nasharuddin Othman). The DWNP's veterinarians and officers treat the wounds on the elephant, as well as provide medication (e.g. Doramectin, Benacillin and multivitamin, see Appendix 7.4), food and water for the elephant before it is released into the forest at a pre-determined location.

The study uses GPS satellite collars made by Africa Wildlife Tracking (South Africa). Each collar consists of three parts: (i) the housing with GPS and battery, that was placed at the back of the neck of the elephant, facing skyward, (ii) the belt that was made from degradable fiber that eventually wears and drops off and (iii) the weights fixed with screws at the bottom of the collar that help to keep the belt tight and the housing stable. The total weight of each collar is approximately 17kg (less than 1% of total body weight of the elephant). For translocated elephants, the collars were placed on the elephants at the release site, towards the end of the translocation process. For free-ranging local elephants at Belum-Temengor Complex and Kenyir Forest Complex, the collars were put in place within approximately 15 minutes after the sedation drug took effect; and the whole process may take approximately an hour. The

revival drug was administered immediately after the collar was secured. This study complied with research and ethic regulations imposed by the Malaysian government (permit: JPHL&TN(IP): 80-4/2) and the Smithsonian National Zoological Park Institutional Animal Care and Use Committee (NZP-IACUC #10-32).

### 3.3.3 Subjects

From March 2013 to April 2015, monitoring of fGCM was carried out on eleven individual elephants and three family groups (see details on animals studied in Table 3-1), but not all elephants provided sufficient data for analysis. Seven individuals were translocated while the rest (individuals and family groups) were local resident elephants at release sites. There were some notable differences in terms of movement and behaviour of translocated elephants compared to local resident elephants. In the beginning, translocated elephants were often by themselves for months after translocation and would avoid crossing the road; while the local elephants have established home ranges, often interact with other elephants and frequently cross the highways (unpublished data, Campos-Arceiz and Wadey).

The seven translocated Asian elephants were captured from various locations around Peninsular Malaysia; four individuals (all males) were released in the Kenyir Forest Complex and three individuals (two males, one female) were released in the Belum-Temengor Forest Complex. The local elephants were divided into two categories: (i) four individual elephants (three males, one female) in Belum-Temengor Forest Complex, and three family groups in Kenyir Forest Complex. The local individual

elephants were elephants that we could identify their dung with confidence (individual repeated measures) for most of the trackings. The local family groups consist of at least five or more individuals that often move together, making it difficult to identify individuals and their respective dung piles, therefore multiple fGCM samples were collected and identified as belonging to the group (repeated group cross-section sampling).

Data from the only female translocated elephant in this study, Jalong, is used separately as a case study to link fGCM (and HPA axis response) to two important events. This elephant was translocated to Belum-Temengor Forest Complex one year prior to the start of this study and remained solitary. Jalong's fGCM monitoring started on Day-341 post-release and stopped on Day-669, after the GPS satellite housing fell from the belt. In the beginning of the study, Jalong exhibited repeated parallel movements along the highway, which was not seen to this degree in other collared elephants in this study (see Figure 3-2, top panel). There were two known and presumably challenging (stimulation of the HPA axis) events during the monitoring of this elephant. The first event was the birth of Jalong's calf that took place shortly before Day-481 post-release; this indicates that she was likely to have been four to six months pregnant when she was translocated. The second event was when she crossed the highway for the first time on Day-537 post-release. After crossing the road, Jalong exhibited more exploratory movements (going into areas previously not explored), and moved further away from the road (see Figure 3-2, bottom panel).

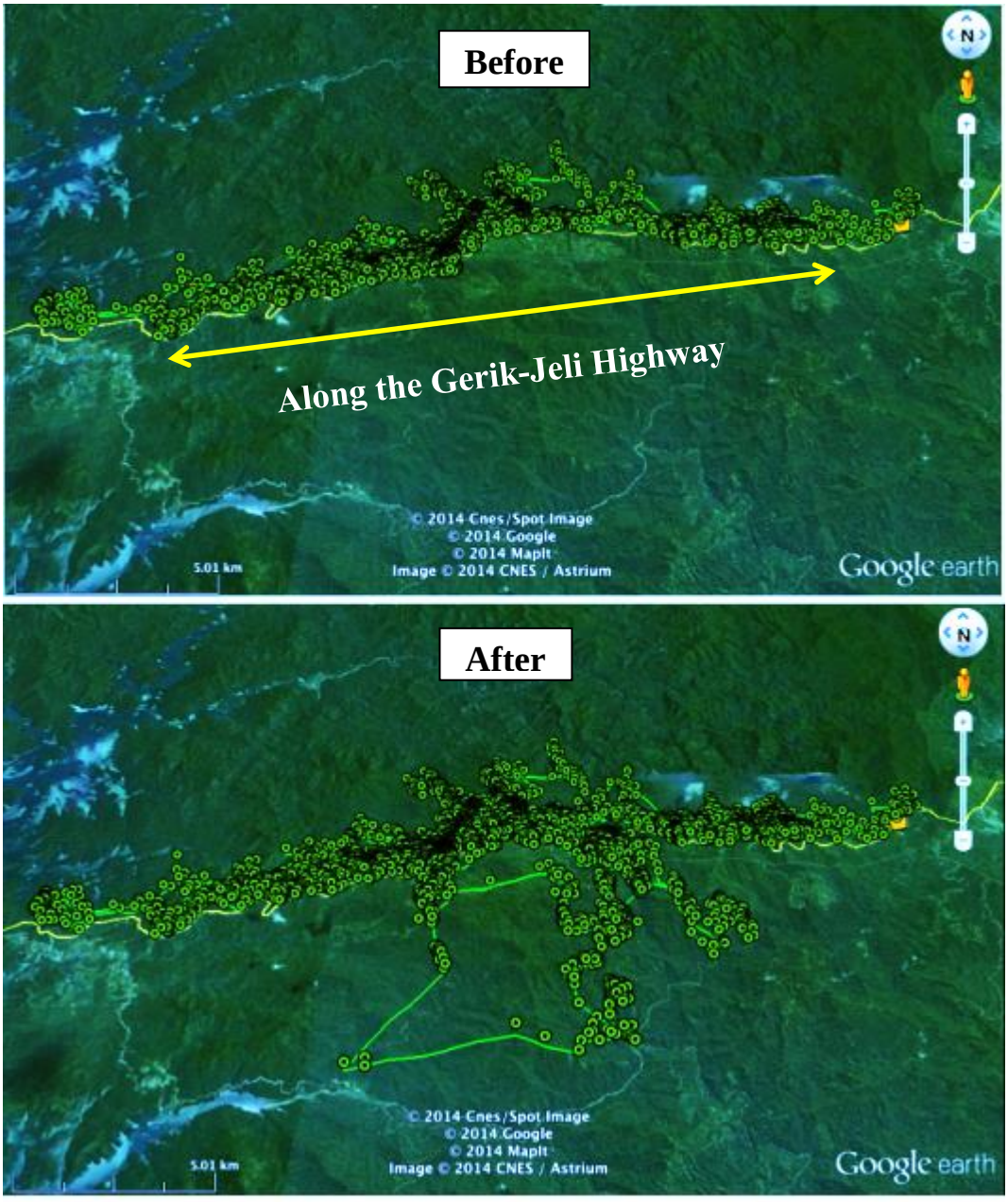


**Figure 3-2: Combined tracklog of Jalong's movement before and after crossing the road on Day-537 post-released.**

**Table 3-1: Elephants' status, age group, location and tracking duration.**

| No. | Elephant Name | Age group | Status [#] | Forest complex | Date of collaring | Duration tracked (days post-release ) | Number of samples (passed QC) * |
|---|---|---|---|---|---|---|---|
| 1. | Puah | NA | Local Family, Group | Kenyir | 14 Apr 2014 | 66-378 | 78 (78) |
| 2. | Sireh | NA | Local Family, Group | Kenyir | 13 May 2014 | 42-321 | 58 (58) |
| 3. | Sulaing | NA | Local Family, Group | Kenyir | 14 Oct 2014 | 14-192 | 29 (29) |
| 4. | Puteri Rafflesia | Adult | Local Individual, Female | Belum-Temengor | 19 Jan 2014 | 0-450 | 25 (14) |
| 5. | Awang Banun | Subadult | Local Individual, Male | Belum-Temengor | 20 Feb 2013 | 62-467 | 39 (19) |
| 6. | Awang Mendelum | Adult | Local Individual, Male | Belum-Temengor | 3 Apr 2013 | 62-320 | 20 (10) |
| 7. | Awang S.Kedah | Subadult | Local Individual, Male | Belum-Temengor | 19 Feb 2013 | 73-536 | 35 (13) |
| 8. | **Mek Jalong** | Adult | Translocated, Female | Belum-Temengor | 20 May 2012 | 341-669 | 26 (13) |
| 9. | **Awang Halim** | Subadult | Translocated, Male | Belum-Temengor | 20 May 2013 | 0-380 | 36 (16) |
| 10. | **Awang Kapak** | Subadult | Translocated, Male | Belum-Temengor | 27 Nov 2013 | 0-184 | 20 (3) |
| 11. | **Awang Tahan** | Adult | Translocated, Male | Kenyir | 27 Oct 2013 | 0-502 | 27 (10) |
| 12. | **Awang Badur** | Subadult | Translocated, Male | Kenyir | 3 Sept 2014 | 0-244 | 24 (21) |
| 13. | **Awang Bakti** | Subadult | Translocated, Male | Kenyir | 17 Oct 2014 | 0-202 | 14 (10) |
| 14. | **Awang Teladas** | Adult | Translocated, Male | Kenyir | 22 Oct 2014 | 0-186 | 13 (10) |

[#] The adults and subadults in this study were differentiated by height: adult male elephants (>15 years old) are the tallest ( >2.4 meters) in comparison to subadult males (5 to 15 years old, 1.8-2.4 meters) and adult female elephant (>15 years old, > 2.1 meters) (Arivazhagan and Sukumar, 2008).

*QC – Quality checked (preserved in freezer below 8 hours after defecation and with high identity confidence)

### 3.3.4 Faecal sample collection

The collection of faecal samples followed this tracking protocol: first, the location of the elephant was determined from the most recent recorded GPS location, the strength of the collar's VHF signal and recent forest signs made by the elephant/s (e.g. footprints, disturbed vegetation and the sound of flapping ears and feeding). When a fresh dung pile from the targeted elephant was located, a record was made of the GPS location, environmental variables associated with the dung pile (i.e. found under sun or in the shade and on vegetation or soil) and social variables (the elephant was in a group or alone).

The faecal samples were collected using clean surgical gloves and stored in a zip-lock bag; approximately 100g of dung was removed from the middle of the bolus from an average of three intact boli (Ganswindt et al., 2010; Palme, 2005). The fGCM samples were mixed thoroughly in the zip-lock bag and placed immediately in a cooler bag with ice packs, before transferring it to a portable car compressor freezer (-15°C to -18°C; Mobicool CF18C and CDF-11, Germany, powered by car AC socket) or chest freezer (-20°C) at the fieldhouse. The field team's goal was to get the samples into the freezer within eight hours after defecation.

For translocated elephants, sampling was carried out more intensively in the beginning of monitoring with four sampling sessions within the first week post-released (see Figure 3-3 for sampling timeline). After sixty days, sampling sessions were reduced to once or twice a month for each translocated elephant. The local elephants (individuals or family groups), which served as controls were monitored

once or twice a month throughout the study after sixty days post-release, comparable to translocated elephants (after sixty days post-release). Sampling for the elephants were terminated either when the GPS tracking collars stopped working or until April 2015.

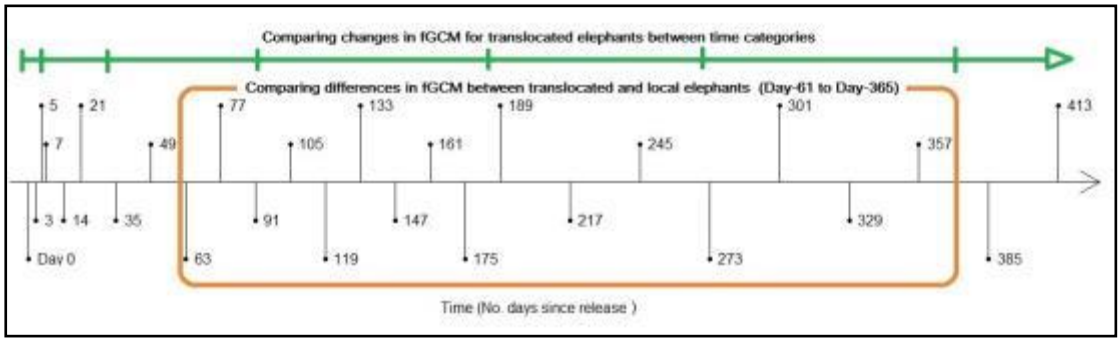


**Figure 3-3: An example of faecal glucocorticoid metabolites (fGCM) sampling session (Day 0 represent the day that translocated or local elephants were collared and released).**

The success in obtaining samples during a sampling session varied, and some sessions had to be repeated a day or two later or cancelled if the elephant was not located (delayed transmission of GPS location points or no points) or if the elephant was located too far (takes more than eight hours to get back to the vehicle). In the Belum-Temenggor Forest Complex, one elephant, Kapak, crossed the border to the neighbouring country, Thailand, and sampling could not be carried out. Sampling sessions were also disrupted during monsoon season (December to February) in 2013 and 2014. Most efforts to obtain faecal samples for fGCM concentrations prior to translocation was unsuccessful for the translocated elephants or from collared local elephants, except for one sample from Puteri Rafflesia (local individual, adult female) collected before collaring event (first sample).

In total, 462 dung samples were collected and analyzed for this study. Only samples estimated to be less than 8 hours from defecation time to freezing time were included in statistical analysis. If post-defecation time for the dung could not be determined in the field (because of thick vegetation foliage, we could not observe when the defecation was taking place), we used the satellite GPS tracking logs to estimate the time the elephant was in the area prior to the sample collection time and made a conservative time estimate (assumed the longest possible duration). For translocated and local individual elephants, these samples were further sorted based on observer's confidence on the identity of the dung. Only dung piles with high confidence of identification (elephant was alone or can be identified from a small group of elephants) were accepted while those with medium or low confidence (elephants were in a group and with no clear observation of the elephant) were discarded. In total, there were 305 samples (Quality Control dataset) that were placed in the freezer within estimated eight hours post-defecation, and with high identification confidence.

### 3.3.5 Hormone analysis in the laboratory

The faecal subsamples were extracted using a wet-weight extraction technique adapted from Walker et al. (2002) and described elsewhere (Edwards et al., 2014; Watson et al., 2013), whereby 0.5 g of faecal matter was extracted with 5 ml of 90% methanol, placed on an orbital shaker overnight, dried and reconstituted in 1 ml of 100% methanol, and stored at -20°C until being analyzed with a corticosterone EIA (CJM006 supplied by Coralie Munro, UC Davis). The assay has been biologically and biochemically validated for use in Asian elephants (Watson et al., 2013). The corticosterone antiserum CJM006 cross-reactivities are published elsewhere (Watson

et al., 2013) and only data with intra-assay coefficient of variation (CoV) of less than 10% and inter-assay CoV less than 15% were accepted and used for statistical analysis.

## 3.4 Data analysis

For statistical analysis, Linear Mixed Models (LMMs) were used to compare repeated measures within individuals, a method that is able to accommodate samples of unequal sizes. The LMMs were run using statistical software R (version 3.1.1; R Core Team, 2014) and the function lmer in the package lme4 (Bates et al., 2014). *P*-values were calculated with a Satterthwaite approximation using the package lmerTest (Kuznetsova et al., 2015). The protocol for selecting a model with optimal fixed effects and random covariates was based on Galecki and Burzykowski (2013) and Zuur et al. (2009), using a stepwise removal protocol with likelihood ratio (LR) tests, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). For all LMM models, we used the log transformed fGCM concentrations (ng/g) as the response variable, to fulfil the requirement of homogeneity and normality for the model's residuals. The final LMM models were compared to the null model (response variable with random covariate only) using LR tests, to test the relevance of the fixed factors. The final model's assumption of homogeneity and normality of residuals were checked using Pearson standardized residuals.

There was insufficient sample size for testing the effect of season, sex (male or female), age group (subadult or adult) and location (Belum-Temengor Forest

Complex or Kenyir Forest Complex). This is partly due to the nature of this study, as we opportunistically collared elephants captured by DWNP and the success rate depends on circumstances in the field (e.g. efforts by different field teams) and decision taken by the DWNP which is beyond the control of this study. Instead, this study focus on monitoring changes within individuals over time whereby, comparison was standardised based on time since collared and release (denote as "time since release") for translocated and local resident elephants.

### 3.4.1 Comparing fGCM concentrations between translocated and local elephants

Translocated elephants were compared to local individuals (repeated measures) and translocated compared to local family groups (repeated group cross-section sampling), separately. For statistical comparison, a subset of samples with 61 to 365 days post-release were extracted from the QC dataset, representing the time period with most overlap between translocated elephants (N=5, 36 dung piles) and local elephants [individuals (N=4, 39 dung piles) and family groups (N=2, 127 dung piles)]. In this comparison, one translocated male (Kapak) and one translocated female (Jalong) from Belum-Temengor Forest Complex were not included as there were insufficient data on them for this timeframe. The subset of this time period also served to minimize the potential short-term effect of stress from collaring for the local elephants, to get a more accurate estimate of their basal fGCM concentrations.

The elephants' identity is the only random covariate in the LMM, because it has the lowest AIC and BIC. The following fixed factors were tested for importance to the model: time since release (days: 61 to 365 days), status (translocated vs. local individuals or local family groups), interaction between time since release and status, microhabitat 1 (dung piles located under sun, shade or unknown) and microhabitat2 (dung piles located on soil, vegetation or unknown). Microhabitat variables were included because the effect of the sun and activity of bacteria in the soil could potentially cause changes in fGCM molecules in the exposed dung piles (Moschet and Hollender, 2009; Snow et al., 2009; Xuan et al., 2008). Although steps were taken to minimize microhabitat influence by analyzing fGCM samples collected within eight hours post-defecation, these microhabitat variables were included to check for their potential influence. To compare if the results of LMM were the same if the monitoring period was split into smaller timelines, the LMM model was retested with time since release as a categorical variable with four time periods (61-90 days, 91-180 days, 181-270 days, 271-365 days).

### 3.4.2 Comparing fGCM patterns among translocated elephants

For statistical comparison among translocated elephants (N=7, 83 dungpiles), LMM with random intercepts (using elephant as random covariate) was used to compare fGCM changes from day of translocation until the end of the monitoring period for each elephant. Two fixed factors were compared: study sites (Belum-Temengor Forest Complex and Kenyir Forest Complex) and time since release (as categorical variable, with six time periods: 0-7 days post-release, 8-30 days, 31-90 days, 91-180 days, 181-270 days, 271-365 days and 366-502 days). To detect differences in fGCM

concentrations between time periods, the dataset with translocated elephants was relevelled with each time period, and the model was rerun.

### 3.4.3 Case study: Jalong

The fGCM profile of the female translocated elephant (Jalong), with known parallel movement beside the highway, was presented in connection with two specific events that may be stressful for her :(i) after giving birth to a calf (occurred shortly before Day-481 post-release) and (ii) crossing the highway for the first time at Day-537 post-release.

### 3.4.4 Possible observer impact on elephant movements

The observer's impact on fGCM is minimal, since immediate changes in hormone concentration in the blood is not reflected in fGCM samples collected during the tracking session. The fGCM present in the dung represent the average biologically active 'free' glucocorticoid concentrations circulating in the body (Sheriff et al., 2010a; Touma and Palme, 2005), for the duration of the gastrointestinal transit time (one to two days in elephants; Ganswindt et al., 2003; Wasser et al., 2000, 1996). However, it is possible that presence of the tracking team during sampling sessions may have affected the elephants' movement. To examine this possibility, a comparison was made of the distance travelled by the elephants a day before, during and after the tracking period, using the non-parametric Kruskal-Wallis test separately for each treatment group: translocated, local individuals and local family groups.

## 3.5 Results

### 3.5.1 Faecal GCM concentrations in translocated and local resident elephants

Between Day-61 and Day-365 after released, translocated elephants had lower basal fGCM values (mean: 8.49 ± 1.88 ng/g (SD)) than the local individual elephants (mean: 11.35 ± 2.75 ng/g (SD); LMM: t=-2.789, df=6.95, *P*=0.027, see Figure 3-4 and Table 3-2). Likelihood ratio comparison shows that LMM with elephant's status (local individuals vs translocated, $\chi^2$=6.69, df=1, *P*=0.01) as the only fixed effect had the smallest BIC. The model did not detect any interaction between time since release and status (translocated or local individuals; $\chi^2$=3.789, df=1, *P*=0.052), microhabitat 1 (under sun, shade or unknown; $\chi^2$=1.1367, df=2, *P*=0.567) and microhabitat 2 (on soil, vegetation or unknown; $\chi^2$=0.4999, df=2, *P*=0.779). Particularly, the time since release as a continuous variable ($\chi^2$=2.67, df=1, *P*=0.10) or as categorical variable ($\chi^2$=5.30, df=3, *P*=0.15) was considered not important to the model; with or without it, the results were similar. The final LMM was significantly different ($\chi^2$=6.71, df=1, *P*=0.01) from the null model. The fGCM concentrations did eventually increase in two translocated elephants (Jalong and Tahan) after more than a year after release, and the increase lasted a few months - up to the end of monitoring period (see Figure 3-4).

Meanwhile, LMM comparison between translocated elephants and local family groups did not detect any important fixed factors that influenced the fGCM pattern (no difference from null model, *P*-value > 0.05).

**Table 3-2: LMM (lmerTest) results for faecal glucocorticoid metabolites (fGCM) translocated vs. local individual elephants**

| Type | Factor | Estimate | Coefficient (s.e.) | Df | t-value | 95% CI | P-values |
|---|---|---|---|---|---|---|---|
| | Intercept (Local) | 1.083 | 0.041 | 15.90 | 26.62 | (0.981, 1.10) | 1.29e-14 |
| Fixed | Translocated (vs. Local) | -0.122 | 0.044 | 7.09 | -2.767 | (-0.203, -0.037) | 0.0274* |
| Type | Variance | Std. Dev. | 95% C.I. | | | | |
| Random | Elephant Identity (overall) | 0.056 | (0.078,0.110) | | | | |
| | Residual | 0.09 | (0.017,0.091) | | | | |

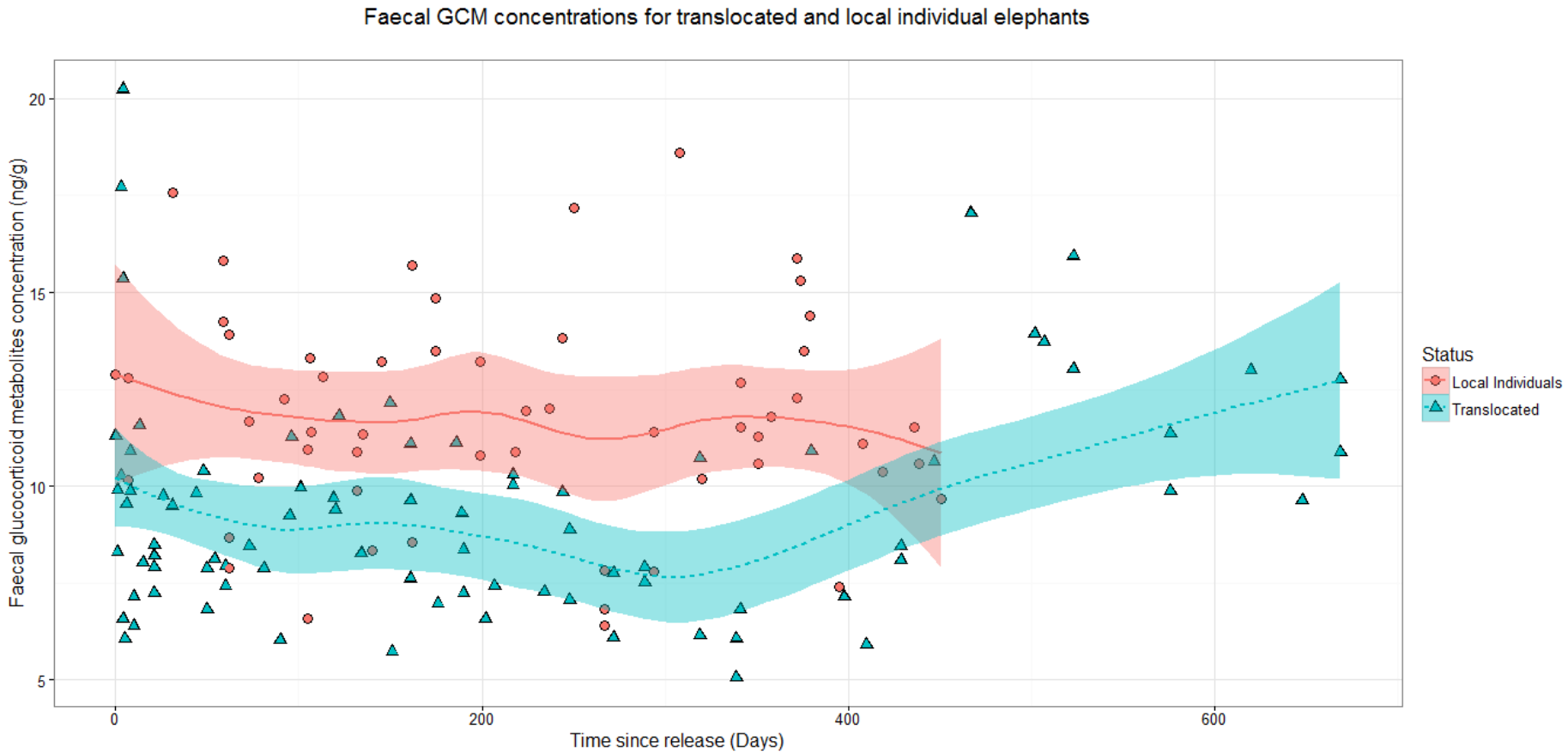


**Figure 3-4: Comparison of overall fGCM concentration between translocated (N=7, green triangles) and local individual elephants (N=4, red circles)**

### 3.5.2 Individual fGCM patterns for translocated elephants

When compared from Day 0 (translocation day) up to the end of monitoring period for each translocated elephants; time since release (as categorical variables, N=7; $\chi^2$=26.61, df=6, *P*=0.0002; Figure 3-5 and Table 3-3); became an influential fixed factor for the LMM. There was no prolonged elevation of fGCM concentrations after release (fGCM was low after the first time period: Day 0-7). The first and last time category (Day 0-7 & Day 366-502) have the largest interquartile range compared to other time periods. Release site did not influence fGCM concentations (Belum-Temengor Forest Complex or Kenyir Forest Complex, $\chi^2$=0.8177, df=1, *P*=0.366). As mentioned earlier, comparison for study site might not be reliable due to small sample size.

**Table 3-3: LMM (lmerTest) comparison of fGCM changes with time since release (categorical time sectors) among translocated elephants.**

| Type | Factor | Estimate | Coefficient (S.E.) | Df | t-value | 95% CI | P-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept duration (0-7) | 1.040 | 0.036 | 25.92 | 28.56 | 0.970,1.106 | <2e-16* |
| | (8-30) | -0.082 | 0.044 | 60.88 | -1.874 | -0.168,-0.0004 | 0.066 |
| | (31-90) | -0.102 | 0.044 | 61.05 | -2.326 | -0.188,-0.020 | 0.023* |
| | (91-180) | -0.063 | 0.042 | 59.80 | -1.506 | -0.142,0.016 | 0.137 |
| | (181-270) | -0.111 | 0.043 | 61.06 | -2.571 | -0.191, -0.027 | 0.013* |
| | (271-365) | -0.231 | 0.050 | 62.85 | -4.621 | -0.325, -0.125 | 1.95e-05* |
| | (366-502) | -0.011 | 0.059 | 61.35 | -0.195 | -0.123, 0.109 | 0.846 |
| Random | Factor | Variance | Std. Dev | CI | | | |
| Elephants' identity | Intercept | 0.002 | 0.045 | 0.001-0.093 | | | |
| Residual | | 0.010 | 0.098 | 0.080-0.113 | | | |

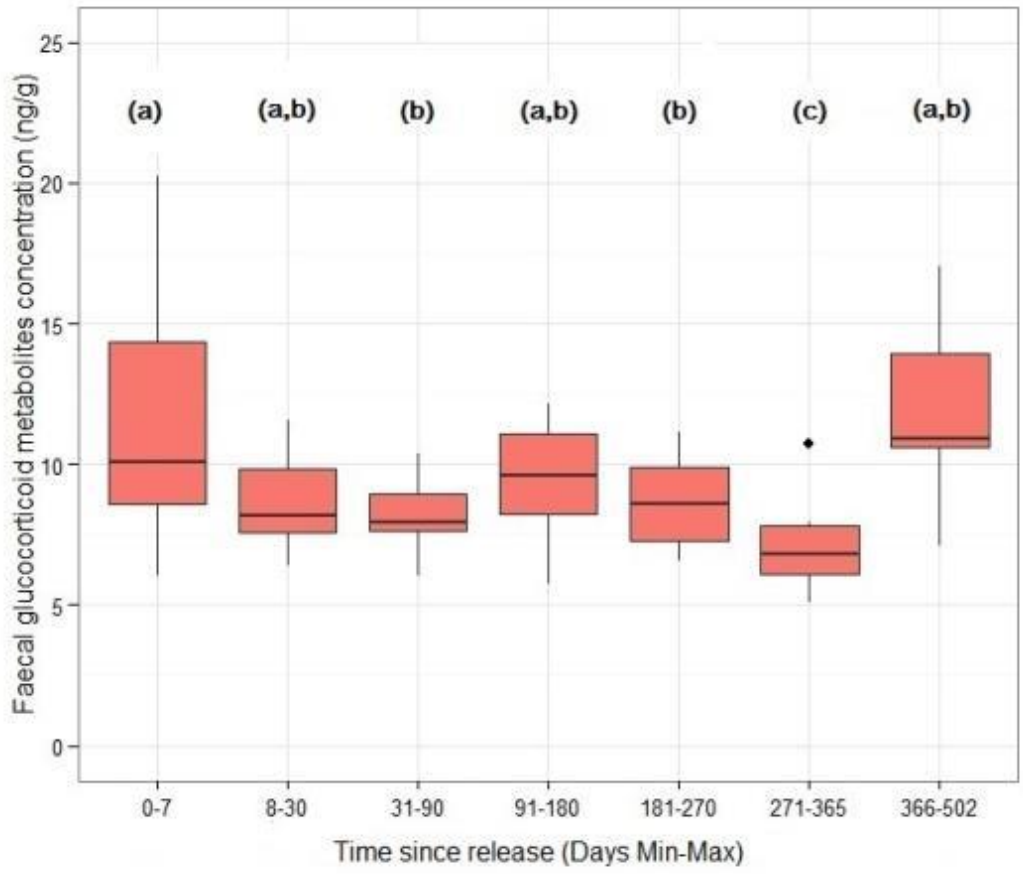


**Figure 3-5: Grouping of translocated elephant's fGCM according to different time period (see alphabet a, b and c that represent the different groups).**

From a descriptive prespective, a translocated elephants individual samples reached some of the highest fGCM concentrations or peaks, in relation to their own profile, from dung samples collected during translocation and tracking within the first week after collaring and release (see Figure 3-6). These peaks were more apparent for elephants in panel 3 to 6 (Figure 3-6, Halim, Kapak, Tahan and Teladas), but it were also true for the first and second elephants (Figure 3-6, Badur and Bakti), except that these two elephants started with low fGCM and did not show a clear drop in fGCM concentration following release. In general the translocated elephants' individual fGCM profiles exhibited two types of patterns: (i) fGCM concentration started low (in

relation to other translocated elephants) and stayed low throughout monitoring period (e.g. Badur and Bakti) or (ii) fGCM started with relatively high values (in comparison to own profile), then showed signs of a drop.

Meanwhile, the lowest fGCM concentrations, in relation to own profile. occurred for the first, fifth and sixth translocated elephant in Figure 3-6 (Badur, Tahan and Teladas) immediately after translocation, and this was also observed within the first week after collaring for local individual elephant, Puteri Rafflesia (the third elephant from right, in Figure 3-7). For the translocated elephants, some of the highest and lowest values of fGCM occurred within the first week after translocation (0-7 days post-release, see Figure 3-6).

### 3.5.3 Case study: Jalong, the elephant who finally crossed the highway

The study started monitoring Jalong at Day-341 post-release and for the first four and half months, Jalong's mean fGCM was 7.31 ±1.18 ng/g (SD). During this time, Jalong was exhibiting repeated parallel movements beside the road (Figure 3-2). Two major events happened after that: Jalong was first observed with a baby at Day-481 post-release (Figure 7-21 in Appendix) and they crossed the Gerik-Jeli highway for the first time at Day-536 post-release. During this time period, Jalong's fGCM fluctuate (mean fGCM: 10.85 ±3.92 ng/g (SD) and Jalong recorded her highest fGCM value shortly before she crossed the highway (15.94 ng/g, highest throughout the monitoring period of eleven months). After crossing the highway, Jalong's movement changed and she began exploring new areas, while the increase in fGCM persisted

(mean fGCM: 11.24 ng/g ±1.41 (SD)) for about four months until monitoring was terminated at Day-669 post-release when the GPS satellite housing detached from the collar (see overall profile at Figure 3-8).

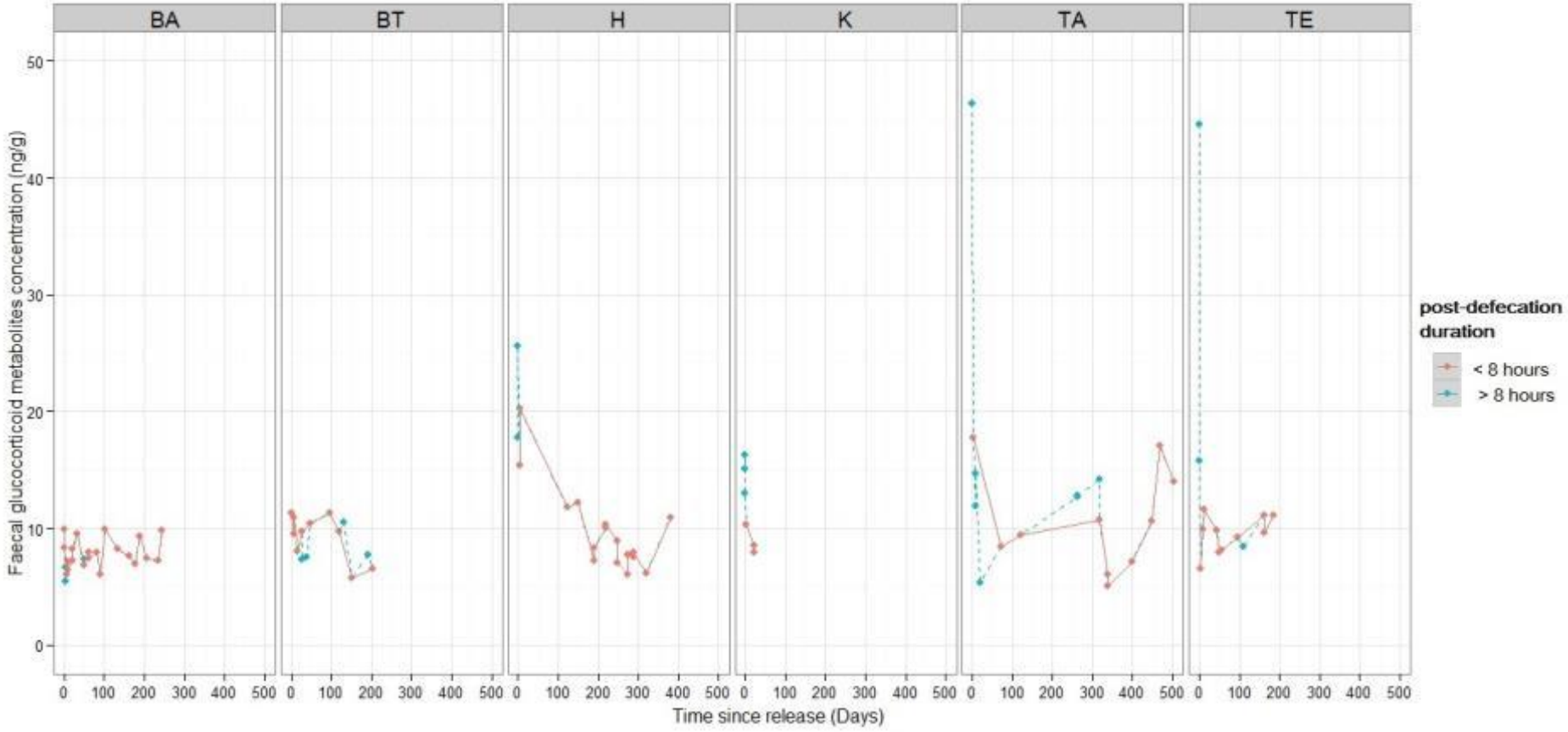


**Figure 3-6: Translocated elephants' faecal glucocorticoid metabolite concentrations (BA=Badur, BT=Bakti, H=Halim, K=Kapak, TA=Tahan and TE=Teladas) aligned from days since collared and released.**

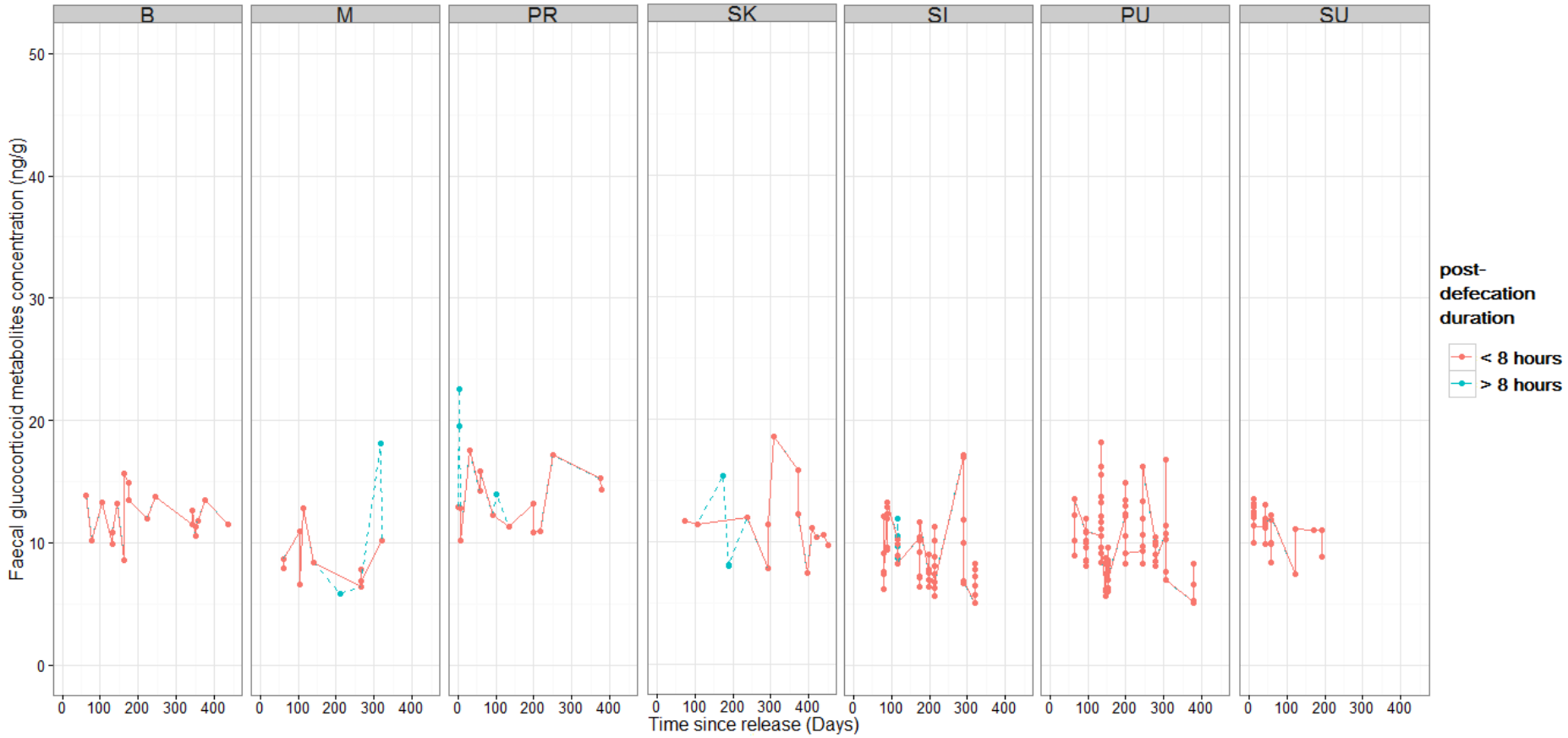


**Figure 3-7: Local elephants' faecal glucocorticoid metabolite (fGCM) concentrations profile (B=Banun, M=Mendelum, PR=Puteri Rafflesia, SK=S. Kedah, SI=Sireh family, PU=Puah family, SU=Sulaing family) aligned from days since collared and released.**

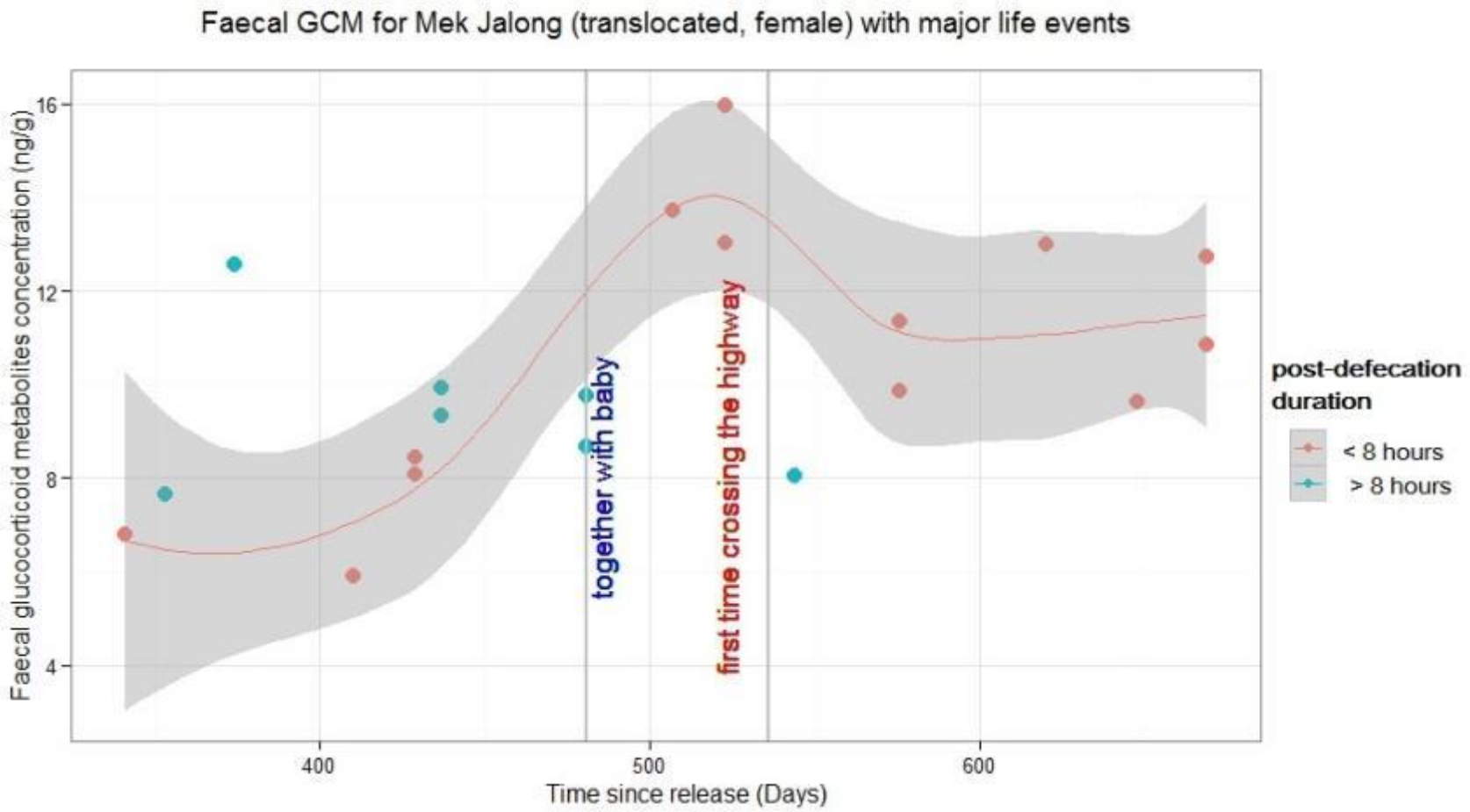


**Figure 3-8: The female translocated elephant Jalong's faecal glucocorticoid metabolites (fGCM) profile with two major events.**

### 3.5.4 Observer effect on elephant movements

There were no significant differences in distance travelled by elephants on the days before, during and after tracking, for each group of elephants [translocated ($\chi^2$=3.2336, df=2, *P*=0.1985) , local individuals ($\chi^2$=1.1432, df=2, *P*=0.5646) and local family groups ($\chi^2$=3.2706, df=2, *P*=0.1949)] when compared with Kruskal-Wallis test. There was no detection of observer effect on elephant movements.

## 3.6 Discussion

This study wanted to compare fGCM profile of translocated wild elephants with wild individual residents and family groups at the release sites. The translocated elephants' exhibited two types of patterns (Figure 3-6): (i) fGCM concentration started low (in relation to other translocated elephants) and stayed low throughout monitoring period (e.g. Badur and Bakti) or (ii) fGCM started with relatively high values (in comparison to own profile) and then dropped. For the first pattern, it is possible that dung piles with high fGCMs were missed during sampling for these elephants; alternatively it could reflect genuine differences in their stress response. Another consideration is the age for most samples collected on Day 0 (translocation) could not be determined and may have artificially elevated fGCM concentrations (see Chapter 2), and therefore were classified conservatively as above eight hours post-defecation (see points in blue, Figure 3-6).

Contrary to expectation, the elevation of fGCM was not prolonged after the release of translocated elephants. Instead, translocated elephants had a significantly lower range of fGCM between two months to a year after release, in comparison to local individual elephants. In addition, "time since release" was not an important variable in the LMM model, which indicates that the fGCM concentrations were stable for both translocated and local individual elephants during that time period. This finding is of concern, as not much is known about the state of prolonged low fGCM and its effect on animal's ability to adjust to new environment (Dickens et al. 2010).

### 3.6.1 Prolonged lower basal glucocorticoids found in other wild-caught wildlife

The state of lowered glucocorticoid after a period of stress has been reported in several other wild-caught animals: African black rhinos (*Diceros bicornis*: Linklater et al. 2010; MacDonald & Linklater 2007), Chukar partridges (*Alectoris chukar*: Dickens et al., 2009) and European starlings (*Sturnus vulgaris:* Cyr & Romero 2007; Cyr et al. 2007). In the black rhinocerous (*Diceros bicornis*) study, the wild caught rhinos initially showed elevated fGCM for 4 to 16 days when placed in captivity. Subsequently, their fGCM concentration fell 2.5 times below precapture baseline and remained low throughout the monitoring phase of 60 days (Linklater et al., 2010). The wild Chukar partridges (*Alectoris chukar)* study found that the stress of temporary captivity followed by translocation had an additive effect; which resulted in prolonged physiological alteration (lasted beyond study monitoring period of 31 days) to the Chukars' HPA axis, with the birds exhibiting lower basal glucocorticoid concentrations, lack of adrenal response to a negative feedback challenge and weight

loss (Dickens et al., 2009a, 2009b). Therefore, chronic stress could result in lowering of basal fGCM, as it was concluded in the European starlings' study as well.

### 3.6.2 Low glucocorticoid concentrations can be detrimental to health

In endocrinology, most studies tend to focus on the negative effects of excess glucocorticoids and seldom examined lowered glucocorticoids and its consequences (Dickens et al., 2009b; Gunnar and Vazquez, 2001; Heim et al., 2000). This is partly due to Hans Selye's legacy that strongly linked stress with an increase in glucocorticoid (Gunnar and Vazquez, 2001; Heim et al., 2000). Low glucocorticoid was not seen as a problem, as the body requires only a small amount of glucocorticoids to fulfil daily requirements, even in human patients suffering from adrenal insufficiency (Bouillon, 2006; Peacey et al., 1997). However, when dealing with stressful situations, too low (insufficient) concentration of glucocorticoids can be detrimental to health. Adrenalectomized cynomolgus macaques (*Macaca fasicularis*), that were given mineralocorticoid supplement and a subphysiological dosage of cortisol (one-tenth of normal cortisol production), suffered physiological instability related to heart and hemodynamic (blood flow), and had higher mortality during and after surgery treatment (Udelsman et al., 1986). In humans, patients with primary or secondary adrenal insufficiency, as well as those exposed to adrenal suppression (due to prolonged or abruptly stopping high dosage glucocorticoid treatment), are vulnerable to 'acute adrenal crisis', a life-threatening condition suddenly triggered by common stressors such as catching a cold (Arlt and Allolio, 2003; Lee and Ho, 2013).

Low basal glucocorticoid conditions was found, as well, in some war veterans and war survivors with Post-Traumatic Stress Disorder (PTSD) (Rohleder et al., 2004; Yehuda et al., 1991), patients with depression (Bremmer et al., 2007) and people living with chronic stress conditions [e.g. financial difficulties (Faresjö et al., 2013), early childhood adversities (Koss et al., 2015; Kraft and Luecken, 2009), caregivers of dementia patients (Leggett et al., 2015) and see reviews (Edwards et al., 2011; Heim et al., 2000)]. Some wildlife studies suggest that PTSD is likely to occur in predator and prey ecology (Clinchy et al., 2013, 2011) and also in African elephants with disrupted social community due to culling or poaching (Bradshaw et al., 2005). As there are evidences in humans, that chronic stress and various other health conditions may reduce adrenal activity and reactivity (Heim et al., 2000), it is important to investigate what are the implications of prolonged state of low glucocorticoid for translocated elephants.

### 3.6.3 Exploring other causes of low glucocorticoid concentrations

It is possible that the translocated elephants did recover from the stress of translocation, and the prolonged low concentration of fGCM in translocated elephants are the results of their HPA axis shifting to a new state of balance (allostasis) as a way of adjusting in their new environment (Fries et al., 2005; McEwen and Wingweld, 2010; Wingweld and Kitaysky, 2002). However, we found two translocated elephants eventually shifted their fGCM range to concentrations comparable to local elephants, which suggests that these two translocated elephants have the capacity to reach a higher range of glucocorticoid responses but did not during the immediate year after translocation. The prolonged state of lower glucocorticoids suggest imbalance in

primary mediators in the body, and more likely the elephants were in a state of allostatic (with physiological "wear and tear" occurring to body and brain), which can be adaptive if within coping abilities of the animal (McEwen, 2004). The cumulative strain of allostatic load can become allostatic overload (state of disease) when overwhelm by other stressors in the environment (McEwen, 2004). To better monitor the allostatic load, there is a need to develop other non-invasive health markers that are able to measure downstream effects of stress on health.

Low glucocorticoids has also been linked with certain behavioural traits or coping styles in wildlife that include aggressiveness, routine formation, lack of behaviour flexibility amongst others (Grand et al., 2012; Koolhaas et al., 1999; Korte et al., 2005); and in humans (males) that include aggression, low harm avoidance, and lack of self-control (Fairchild et al., 2008; Oosterlaan et al., 2005; Platje et al., 2013; Popma et al., 2007; Shoal et al., 2003). Low glucocorticoid may not be the cause of aggression per se, instead it is suggested that higher cortisol helps in exerting 'self-control' (Korte et al., 2005; Shoal et al., 2003) and moderate overt aggression in individuals with high testosterone (Popma et al., 2007; Terburg et al., 2009). Although individual differences (Cockrem, 2013) could potentially explain the differences in fGCM between translocated and local individuals, but this is unlikely considering that (i) all translocated elephants have consistently showed lowering of fGCM after translocation, and that (ii) translocation itself is a very stressful event that would override any individual or personality differences (Sapolsky, pers. comm.). Instead, future study needs to examine if the state of lowered glucocorticoid and combination of high testosterone are indicators for aggressive behaviour in elephants.

Another possible interpretation is that local elephants, who often interact with other elephants and access areas with high anthropogenic disturbance (e.g. roads, villages, or dams) require vigilance (Tsigos and Chrousos, 2002), and the need for a greater (more flexible) adrenal response. In comparison, translocated elephants, who are often alone and may have actively avoided potential stressors, could be experiencing under-stimulation of adrenal activity (Korte et al., 2007; Sapolsky, 2015; Tsigos and Chrousos, 2002). Indeed the case study of Jalong, the translocated female elephant in the current study, who was showing repeated parallel movements next to a highway (attraction to the highway, but the avoidance to cross) is a good example of low adrenal activity prior to the presences of a baby and the challenge of crossing the highway (Figure 3-8 and in Appendix, Figure 7-21).

When this female (Jalong) crossed the highway for the first time there was increase in fGCM concentrations. Elevation in adrenal activity may trigger dispersal, as seen in some juvenile birds or migration in salmons (Korte et al., 2005). However it should also be considered that the first road crossing also coincided with the female giving birth which could also result in an increased in fGCM concentrations (Brown 2000; Walker pers. comm.) The elevation in fGCM that persisted after Jalong crossed the highway was within the usual fGCM range for local elephants in the area who cross the highway regularly. It is not likely that the change in Jalong's fGCM is caused by a change of diet when exploring new areas, since the increase in fGCM occurred even before she crossed the road for the first time. The fGCM elevation for Jalong after crossing the road, should also be considered in relation to the challenges of exploring a new environment (change in movement pattern) and the need to be vigilant with an offspring (Rees et al., 2004). In the future there is potential to explore this idea further

through movement behaviour analysis via GPS tracking combined with the usage of fGCM (Jachowski *et al.*, 2013b).

### 3.6.4 Future research and management implications

In terms of sampling methodology, comparison between known individuals (repeated measures) was more effective in detecting significant differences than family group (repeated group cross-sectional sampling). The fGCM variances in family group samplings were large, and made it difficult to detect important variables in the LMM. In addition, the interpretation of whether fGCM is high or low is relative to the individual's profile (Cockrem, 2013). For future studies of fGCM, we would recommend the use of individual monitoring, behaviour observation and contextual interpretation of events that happened to the individual to help in interpretation of fGCM (Madliger and Love, 2014).

One limitation of this study was the trade-off between the effort to monitor changes in individual fGCM's and the number of elephants that can be monitored (sample size). The small sample size in this study did not allow sufficient comparison between other variables such as male and female, study sites and season. Since this is a field study (uncontrolled environment) and the subjects of this study were obtain by opportunistic chance to capture and collar wild elephants by DWNP's field team, we are unable to dictate these variables, However, monitoring of known individuals helped to increase confidence in the actual status of fGCM within the translocated elephants, and in detecting significant differences from local resident individuals. In future, when cost of processing DNA samples becomes more affordable, DNA identification can be

carried out in tandem with faecal endocrinology to increase identification of individuals belonging to groups. The results of this study is supporting the evidence that free-ranging wildlife may experience lowering of basal glucocorticoids due to chronic stress, nonetheless, either a bigger sample size or a better understanding of what is happening physiologically (to the HPA axis) in these translocated elephants are needed, before the results from this study can be extrapolated to determine the potential effect of translocation on other Asian elephants.

What is also crucial is to consider the optimal concentration of glucocorticoids for health and the "inverted-U" effect of glucocorticoids on cognitive and other neurobiological processes (Korte et al., 2007; McEwen et al., 2015; Sapolsky, 2015). Instead of restricting research interpretation on the high end of glucocorticoids and diseases spectrum, researchers should also consider the effect of low glucocorticoid concentrations on health and behaviour. Without the recognition that low glucocorticoids could be a potential health problem for wildlife, there will be no effort in helping to ameliorate the situation. As example, in human patients, medical doctors recognised the importance of immediate administration of glucocorticoids for acute adrenal crisis (Lee and Ho, 2013), and researchers are examining glucocorticoid therapies for PTSD war veterans rehabilitation (Jia et al., 2015; Suris et al., 2010; Yehuda et al., 2014), and in preventing PTSD development from occurring in patients treated at intensive critical unit (Schelling et al., 2004).

There is a need for non-invasive diagnosis technique for adrenal insufficiency and PTSD in free-ranging wild forest elephants. The current diagnosis procedure for adrenal insufficiency are invasive and require adrenocorticotropic hormone (ACTH)

or dexamethasone (DEX) injections and monitoring of glucocorticoid concentrations changes in the blood (Arlt and Allolio, 2003; Marik and Zaloga, 2002). Meanwhile, for PTSD diagnosis in elephants and other wildlife, often require extensive background record on causes of trauma and behaviour observations (Bradshaw et al., 2005, 2008; Bradshaw and Schore, 2007; Rizzolo and Bradshaw, 2016), all which may not be possible for wild forest elephants. Therefore, a potential area for future research is to adapt hormone readings, animal movement or other non-invasive data to diagnose the symptoms of PTSD (and other psychology disorders) for application on wild elephants in the forest, where there are less observation opportunities due to challenging environment. Currently, neuroscience research laboratories are already using combination of chronic and acute stressors (e.g. exposure to predator, social hierarchy disruption, swimming test, restraining test or electric shocks) to obtain behavioural and neuroendocrinology changes in laboratory rats comparable to PTSD symptoms in human patients such as low basal glucocorticoid, higher sensitivity to DEX, freezing behaviour, avoidance behaviour, fear-conditioned memory and anxiety (Stam, 2007; Zoladz et al., 2012).

In terms of management of wild elephants, conservation authorities and other relevant stakeholders have to consider that the problem does not end at the release site. Translocated elephants undergo physiological changes that could last more than a year. we speculate that with prolonged lower glucocorticoid concentrations, the elephants could be vulnerable because of an altered response to stressors and an inability to react to danger (Dickens et al., 2010, 2009a; Koolhaas et al., 1999). In terms of behaviour, they may exhibit extreme behaviours such as increased aggressiveness or avoidance behaviour (Koolhaas et al., 1999; Popma et al., 2007;

Terburg et al., 2009). In Belum Forest Complex, we found evidence that translocated elephants are less likely to cross the roads in comparison to local resident elephants (unpublished data, Campos-Arceiz and Wadey). Further research is required to monitor and confirm the effect of physiological changes in translocated elephants, and we would recommend post-translocation monitoring up to two years or more and to investigate ways to reduce the impact of stress on HPA axis. Even then, based on precautionary principles and existing evidence, we are ready to recommend to the management authorities to avoid translocation where it is possible and instead explore other HEC mitigation techniques that promote co-existence of humans and elephants in the same landscape.

## 3.7 Conclusion

In this study, we found that translocated elephants did not experience a prolonged elevation in glucocorticoid concentrations after translocation. Instead, the translocated elephants had lower basal fGCM in comparison to local individual elephants. Not much is known about the effect of a prolonged lower state of fGCM on wildlife health, whether it is a sign of adaptation to the new environment or something more physiologically and psychologically severe, as it can be in humans. When assessing HEC mitigation effectiveness, conservation authorities and relevant stakeholders should take into consideration that translocation have a pronounced and long-term effect on translocated elephants' HPA axis. Since translocated elephants' HPA axis response are altered (low basal glucocorticoid concentrations) and they may have adjusted ability to respond to challenges in the new environment. Therefore we recommended that monitoring of translocated elephants to continue up to two years or

more after release at new sites whenever it is possible to do so. For future research on non-invasive monitoring techniques, there is a need to develop more indicators to diagnose health conditions (e.g. adrenal insufficiency, PTSD, allostatic overload) in free-ranging wildlife.

# 4 Relationship between faecal glucocorticoid metabolites, gastrointestinal parasite egg counts and ciliate counts in translocated and local wild Asian elephants

## 4.1 Abstract

Glucocorticoids, one of the hormones produced by the hypothalamic-pituitary-adrenal axis in response to stressors, play a role in modulating immune defences in the body. Stress may result in suppression of the immune system and allow reinfection, a higher establishment of gastrointestinal parasite, and subsequently a higher shedding of gastrointestinal parasite eggs. In this study, to detect signs of immunosuppression, we have carried out monitoring of faecal glucocorticoid metabolites (fGCM), as well as counts of gastrointestinal nematode (strongyles) eggs, trematode eggs (i.e. *Paramphistomatid, schistosomes*) and gut microflora ciliates in five wild male Asian elephants which were captured and relocated to new forest sites in Peninsular Malaysia. For comparison, four local individual elephants and three local family groups were also monitored at the release sites. There were no detection of immunosuppression and no differences in parasite egg counts and ciliate counts between translocated and local elephants. The fGCM concentrations were associated with strongyles egg counts (quadratic relationship) only in translocated elephants. While, *Paramphistomatid* trematode egg counts were found to be positively correlated to ciliate counts, and the pattern was only apparent when comparing individual repeated measures and not group cross-section sampling. We are unable to detect signs of immunosuppression possibly due to various factors such as anti-

parasitic medicine application for some of the translocated elephants, environment, and social factors that need to be taken into consideration in future research.

**Keywords:** Asian elephant (*Elephas maximus)*; faecal glucocorticoid metabolites (fGCM); non-invasive monitoring; nematode, trematode, ciliates adrenal activity; translocation, wild elephants

## 4.2 Introduction

### 4.2.1 Parasites and immune system

Parasitism is considered one of the important but often neglected inter-species interactions (Gomez and Nichols, 2013). Parasites siphon away nutrients from their hosts and can affect their hosts' population size negatively by influencing their hosts' survival, reproduction, behaviour, health, and fitness; (Anderson and May, 1978; Pedersen and Greives, 2008; Thomas et al., 2006; Wilson and Cotter, 2013). To keep parasites and other pathogens (e.g. helminths, protozoa, fungus, viruses and bacteria) at bay, the host's body has to maintain a robust innate immune system which consist of cellular immunity and humoral immunity; and build an acquired-immunity, which allows the immune system to 'remember' past infections and attack similar intruders in future (Allen and Maizels, 2011; Wilson and Cotter, 2013). The humoral immunity is generally regarded as the immune response against extracellular parasites like helminths (Zhu & Paul 2008), but a balance of both cellular (mediated by T helper cell Type 1, Th1) and humoral immunity (mediated by T helper cell Type 2, Th2) is needed to keep parasite infections in check. Over-emphasis on either one type of

immune response could result in autoimmune-diseases or development of allergies (Maizels & Yazdanbakhsh 2003; Maizels et al. 2004). The immune system is an expensive investment by the body in terms of energy and nutrients, that could otherwise have been used for physical growth, reproduction and other life-history traits (Colditz, 2008; Greer et al., 2005; Wilson and Cotter, 2013).

How parasites can infect a host is largely influenced by (i) host population density (higher density increases infection risk), (ii) host's immunity (lower immunity, higher infection), (iii) host's diet (some herbivores may consume plants containing natural anti-parasitic proprieties) and (iv) host's home range (animals with larger home range have a higher diversity of parasites; Lisonbee et al., 2009; Navarro, 2004; Thomas et al., 2006; Watve and Sukumar, 1995). However, for animals that live in social groups, like elephants, transmission may depend on frequency of contact instead of population density. If the social associations are wide enough, parasites or other pathogens may potentially infect the whole population in the area (Thomas et al., 2006).

A popular non-invasive method to study parasite infections is the examination of parasite eggs and larvae using microscope. This method is widely used for study of parasites in domestic animals (McKenna, 1981; Nielsen et al., 2010a; Rieu et al., 2007), free-ranging wildlife (Muehlenbein, 2006; Sak et al., 2013; Seivwright et al., 2004) and humans (Sinniah, 1982; Sithithaworn et al., 1991). Faecal parasitology techniques also enable the study of microflora ciliates. Not much is known about microflora ciliates other than their commensal role in digestion of nutrients, and that none has been found to be pathogenic for elephants (Fowler and Mikota, 2006).

### 4.2.2 Glucocorticoids, stress, and parasites

Glucocorticoids (cortisol and corticosterone) are steroid hormones regulated by the hypothalamic-pituitary-adrenal (HPA) axis, that play a role in modulating energy requirements for daily activities and in managing physiological responses towards stressors (Sapolsky et al., 2000; Tsigos and Chrousos, 2002). Since maintaining the immune system is costly during stressful events, the HPA axis through glucocorticoids may modulate immune activities to reinvest the energy for "fight or flight" (Greer et al., 2005; Martin, 2009; Sapolsky et al., 2000). The intensity, duration and type of stressors will exert different influence on the immune system (Hamilton, 1974; Martin, 2009). In humans, acute stressors will increase innate immunity but compromise acquired-immunity; intermediate stressors (e.g. exams) suppresses cellular immunity but preserves humoral immunity; while chronic stressors (e.g. trauma or loss) may suppress both cellular and humoral immunity (Segerstrom and Miller, 2004).

Adult animals with matured immune systems usually do not exhibit strong correlation between gastrointestinal nematode parasite egg counts and infection intensity (McKenna, 1981; Stear et al., 1995), as the immune system will exert a density-dependent effect on parasite's larvae establishment, growth and fecundity (Paterson and Viney, 2002). Animals with high gastrointestinal nematode larvae infection may have lower parasite egg counts due to longer prepatent period (parasite larvae take longer to mature and have lower fecundity) (Christensen et al., 1996; Nansen and Roepstorff, 1999; Stear et al., 1995). In contrast, animals that harbour a few adult

parasite worms, may escape the immune system's detection and produce a lot of eggs (Christensen et al., 1996; Nansen and Roepstorff, 1999; Stear et al., 1995).

However, animals with compromised or undeveloped immune system will not be able to exert density-dependent effect on gastrointestinal nematode parasites and these animals may show higher correlation between worm burden and egg counts (Greer et al., 2005; Paterson and Viney, 2002). Experiments with hormone manipulation showed that application of glucocorticoids can suppress innate and acquired-immune resistance in the hosts. This results in the hosts' inability to reduce the establishment of infective larvae in the gut through secretion of mucus and expulsion of larvae (Huntley et al. 1992; Douch et al. 1994; Peña et al. 2004); thus increases parasite infections and production of parasite eggs (Greer et al. 2005; LaFonte & Johnson 2013; Hamilton 1974; Paterson & Viney 2002). One study by Foley et al., (2001), found five out of 16 free-ranging African female elephants in their study had large numbers of white nematode worms in their faeces, and exhibited the highest level of fGCM throughout 23 months of field monitoring.

### 4.2.3 Translocation and parasites egg counts

Translocation of elephants from human-elephant conflict areas to new forest habitats is a stressful event, as the animal is restrained during translocation and separated from its family or bachelor groups. Translocated elephants will have to face challenges in adapting to a new habitat with presences of unknown elephants or find their way home (Choudhury, 1993; Fernando et al., 2012). The hypothesis of this study is that elephants undergoing translocation will experience stress from this activity, and may

experience a weaker immune response (immunosuppression), and gastrointestinal parasites may therefore increase in quantity. Previously, transportation and confinement of sheep was found to increase egg counts of lancet liver fluke (*Dicrocoelium dendriticum*) in faecal samples and possibly disrupted the ability of the immune system to naturally reduce larvae load in comparison to sheep in field (Sotiraki et al., 1999).

An existing wild Asian elephant translocation activity in the Department of Wildlife and National Parks (DWNP), Peninsular Malaysia, and satellite GPS collaring project under Management & Ecology of Malaysian Elephants (MEME), provides the opportunity to track known free-ranging translocated elephants and local resident elephants (individuals and family groups). The objective of this study is to compare quantification of parasite eggs and microflora ciliates, and faecal glucocorticoid metabolite concentrations between translocated and local elephants, to detect if there is an effect of immunosuppression caused by the stress of translocation (high glucocorticoid concentrations and high number of parasite egg counts; Greer et al., 2005). If immunosuppression can be detected using faecal parasitology, it can be developed into an important non-invasive monitoring indicator for measuring the downstream effect of stress on the immune system. Nonetheless, the relationship between glucocorticoids and quantification of gastrointestinal parasites eggs may not be direct, as delayed effects between the time of infection and prepatent period may confound the pattern (pers. comm. Modry).

There is very little study on parasites for wild free-ranging elephants in Malaysia, and most published parasitology studies were on the captive population (Caple et al.,

1978; Fowler and Mikota, 2006). In 2013, a parasitological study carried by Hing et al. (2013) on free-ranging wild Bornean elephants was considered the first of its kind.

## 4.3 Materials and Methods

This study complied with research and ethic regulations imposed by the Malaysian government (permit: JPHL&TN(IP): 80-4/2) and the Smithsonian National Zoological Park Institutional Animal Care and Use Committee (NZP-IACUC #10-32).

### 4.3.1 Study Area

The study area for this project included the two largest forest complexes, Belum-Temengor Forest Comples and Kenyir Forest Complex in northern Peninsular Malaysia (Hedges et al., 2015; Rayan and Linkie, 2016). Both areas are dipterocarp forests with a matrix of land use including protected areas, forest reserves with permitted logging, local villages, plantations, lake, rivers and dams; and its forests are bisected by a major highway (see Figure 3-1).

### 4.3.2 Translocation procedure and collaring

The process of capturing and translocation of wild elephants is managed by the Department of Wildlife and National Parks (DWNP) according to standard operating procedure described in Daim (1995). The translocation process for an elephant may take from three days to a week. Translocated elephants were usually given anti-parasitic medication (e.g. Doramectin) before they were released. See Appendix 7.4

for the list of additional medication and supplements given to translocated elephants. However, these drugs were not given to the local elephants.

The study uses GPS satellite collars (~ 17kg) made by Africa Wildlife Tracking (South Africa). For translocated elephants, the collars were placed on the elephants at the release site, towards the end of the translocation process. For free-ranging non-translocated elephants, the collars were put in place within approximately 15 minutes after the sedation drug took effect; and the whole process may take approximately an hour.

### 4.3.3 Subjects

Elephants and dung samples used in this study is a subset from a larger study on faecal glucocorticoid metabolites (fGCM) carried out from March 2013 to April 2015 (see Chapter 3). The subjects for this study are five translocated male elephants, three local resident males, one local resident female and three local family groups (see Table 4.1 for details). The male and female elephants are individuals that we could identify their dung with confidence (individual repeated measures) for most of the trackings; while the local family groups consist of at least five or more individuals that often move together, making it difficult to identify individuals and their respective dung piles (repeated group cross-section sampling).

### 4.3.4 Faecal sample collection

The fGCM samples were fresh elephant faeces collected from the middle of the dung bolus from at least three different dung boli, and these samples were thoroughly mixed in a clean zip-lock bag and then freeze as soon as possible. Sampling protocol for faecal parasitology samples are as follows: a subsample (three small pieces of faecal samples) were taken from the middle of the dung bolus, and stored in a 50ml Falcon tube with 30ml 4% formaldehyde (10% formalin) for parasite analysis.

### 4.3.5 Hormone analysis in the laboratory

The faecal subsamples were extracted using a wet-weight extraction technique adapted from Walker et al. (2002) and described elsewhere (Edwards et al., 2014; Watson et al., 2013), whereby 0.5 g of faecal matter was extracted with 5 ml of 90% methanol, placed on an orbital shaker overnight, dried and reconstituted in 1 ml of 100% methanol, and stored at -20°C until being analyzed with a corticosterone EIA (CJM006 supplied by Coralie Munro, UC Davis). The assay has been biologically and biochemically validated for use in Asian elephants (Watson et al., 2013). The corticosterone antiserum CJM006 cross-reactivities are published elsewhere (Watson et al., 2013) and only data with intra-assay coefficient of variation (CoV) of less than 10% and inter-assay CoV less than 15% were accepted and used for statistical analysis.

### 4.3.6 Parasitology analysis in the laboratory

Dung sample for parasitology was processed together with its formaldehyde immersion in the laboratory. We used a ceramic mortar and a pestle to homogenize the sample and formaldehyde, together with some distilled water. Then the homogenized solution was strained (to remove fibre and large items) and placed into a clean 50ml Falcon tube (that had its weight pre-recorded). After that, the samples were centrifuged at 2000 rpm for 5 min, and then the supernatant (water and formaldehyde) were poured off, and the weight of the remaining wet sediment was recorded for each sample. Finally, 10 ml of 4% formaldehyde (10% formalin) was added to each sample and the sample was stored for subsequent analysis (Červená, 2013).

To confirm the presences of parasite eggs or microflora ciliates in an individual, usually three to four dung samples per individual were sufficient (Muehlenbein, 2005; Pomajbíková et al., 2012). Time after defecation maybe of importance to the development of parasite eggs; strongyles egg counts in captive black rhino's dung were stable up to six hours and declined nine hours after defecation (Stringer et al., 2014), while faecal egg counts in horse dung were stable up to twelve hours (Nielsen et al., 2010b). In this study only samples below eight hours were included in the statistical analysis.

Gastrointestinal nematodes from the family Strongylidae, produce recognisable eggs with clear oblong shells, but which are difficult to differentiate morphologically to species levels. Instead they are usually categorised together as "strongyles" (Fowler

and Mikota, 2006). Trematodes (also known as flukes) under the family *Paramphistomatid* (with snails as intermediate hosts) produce large oval eggs with operculum on one end of the egg (a cap structure on the egg with hinges, which opens like a door). On the contrary, the blood flukes (schistosomes), for example *Bivitellobilharzia nairi*, has a spine or hook at one end of the egg (Agatsuma et al., 2004; Devkota et al., 2012; Rajapakse et al., 2013). The methods for examination of parasite eggs can also be used for examining microflora ciliates from the gastrointestinal tract, and the ciliates are recognisable with iodine staining and their little tuffs of cilia (Červená, 2013; Kinsella et al., 2004). See Appendix 7.5 for the pictures of strongyles, trematode eggs and gut microflora ciliates under the microscope.

For quantification of parasite eggs and ciliates under the microscope, the processed samples (10 ml, 4% formaldehyde) were hand-shaken (inverted approximately ten times) to mix-up the sediment and formaldehyde well, before a 200µl amount was transferred via pipette into a 1.5ml Eppendorf tube and approximately 1.3ml distilled water was added. The sample in Eppendorf tube was centrifuged at 1500 rpm for three minutes. The top layer of the supernatant was carefully removed via pipette and the remaining sample was mounted on a microscope slide with a drop of iodine (Dobell & O'Connor Iodine, R21510, Remel BactiDrop for intestinal protozoan). All strongyles eggs, trematode eggs (distinction made between *Paramphistomatid* and blood fluke) and microflora ciliates on the entire slide were counted at 100× magnification with the microscope (Červená, 2013). The counts were then extrapolated for the whole 10ml as standardised count per gram (CPG) of wet weight

sediment with the following equation: [(50 × total count in 200ul)/ wet weight of sediment].

## 4.4 Data analysis

For statistical analysis, Linear Mixed Models (LMM) were used with the statistical software R (version 3.1.1; R Core Team, 2014) and the function lmer in the package lme4 (Bates et al., 2014). The protocol for selecting fixed effects and random covariates was based on Galecki and Burzykowski (2013) and Zuur et al. (2009), using a combination of likelihood ratio (LR) tests, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) (Zuur et al., 2009). The *P*-values were calculated with a Satterthwaite approximation using the package lmerTest (Kuznetsova et al., 2015).

For individual translocated elephants and individual local elephants, only samples with high elephant identification confidence (elephant was alone or can be distinguished from other elephants in a small group) were included in statistical analysis (individual repeated measures). For local family groups, since it is not possible to identify dung piles of individual elephants, samples were collectively identified as a group (group cross-section sampling). Because of the differences in representation of the samples between individual local elephants and local family groups, these two groups were compared with translocated elephants separately in data analysis.

Respective parasites' egg counts per gram (CPGs) and ciliate CPGs were transformed with $\log_{10}(x+1)$ to fulfill the LMMs' requirement of homogenous and Gaussian distributed residuals. For LMM tests, the random covariates with the smallest AIC and BIC were 'elephant identity' (random intercept) or 'elephant identity' with auto-correlation with 'time since release' (random intercept with random slope). There was difficulty to run LMM models with random covariate (random intercept with random slope) due to small sample size, so instead 'elephant identity' (random intercept) was used as the standardised random covariate for all LMMs (Zuur et al., 2009).

The LMMs were used to analyse strongyles egg CPGs, trematode egg CPGs and ciliates CPGs (one at a time) and their relationship with the following fixed factors: (i) time since release (days: continuous or categorical), (ii) Status (translocated vs. local individuals or local family groups), (iii) average boli circumference, (iv) Microhabitat 1 (dungpile under shade, sun or unknown), (v) Microhabitat 2 (dungpile on soil, vegetation or unknown), (vi) study site (Belum-Temengor or Kenyir), (vii) faecal glucocorticoid (fGCM) concentrations (ng/g, linear and quadratic), (viii) interaction between 'Status' and 'fGCM concentrations (linear and quadratic)' and (ix) interaction between 'Status' and 'time since release'. In addition, strongyles egg CPGs, trematode egg CPGs and ciliates CPGs were included as fixed factors for each other (in turns), as relationships between them may reflect conditions inside the body or sampling conditions (as they were from the same sample obtained in the field).

The average boli circumference measurements can be used as a proxy for the animal's size instead of categorical age group (Jachmann and Bell, 1984; Morrison et al., 2005; Nowak et al., 2009). Boli measurements for elephant age group may differ due to diet,

between different populations or in captive conditions (Hedges, 2012; Reilly, 2002), but for the purpose of this study the boli measurements served as a comparative reference for age and physical size between the elephant subjects in this study.

Microhabitat 1 (under sun, shade and unknown) and Microhabitat 2 (on soil, vegetation or unknown) were local conditions at the dung piles location. Different microhabitat conditions may affect parasite eggs; for example, there could be more dung beetles in shaded area with soil which could affect development of parasite larvae (Bryan, 1973). However, the effect of environment on parasite egg counts should be low since fresh dung samples were collected from the middle of the bolus (Stringer et al., 2014). The two study sites (Belum-Temengor Forest Complex and Kenyir Forest Complex) were included to check if there were any spatial differences although the sample size is small.

The linear and quadratic terms for fGCM concentrations in the samples were included as fixed factors, considering that glucocorticoids may have a biphasic (strengthening or weakening, depending on the hormone's concentration effect on mineralocorticoid and glucocorticoid receptors) influence on physiological changes in the body including the immune system and health (Chrousos, 1998; Silverman et al., 2005). Based on previous analysis on fGCM concentrations (discussed in chapter 3), we found consistent fGCM differences between translocated and local individual elephants from Day-60 to Day-365 post-release. Therefore the interaction between the elephant's status and fGCM (linear and quadratic) were included as part of the fixed factors to be tested.

### 4.4.1 Comparing parasite eggs and ciliates (Count Per Gram) between translocated and local elephants

The LMMs with random and fixed factors listed in section "4.4 Data analysis" were used in the comparison between translocated and non-translocated individuals (local individuals or local family groups); for time period between Day-61 and Day-365 (continuous variable). The gap of sixty days was chosen to reduce the effect of collaring on non-translocated individual elephants and this time period have the highest overlap among translocated elephants (N=5, 28 samples), non-translocated elephants (local individuals (N=4, 28 samples), and family groups (N=3, 52 samples) in this study.

### 4.4.2 Comparing parasite eggs and ciliates (Count Per Gram) among translocated elephants

Random covariates and fixed factors used in LMM analysis were as described under "4.4 Data analysis", except that post-release duration was analysed as a categorical variable with five time periods: Day 0-90, Day 91-180 days, Day 181-270, Day 271-365 days and Day 366 -502 days; to detect changes in parasite egg counts and ciliate counts within the monitoring period.

## 4.5 Results

### 4.5.1 Comparing parasite eggs and ciliates (Count Per Gram) between translocated and local elephants

The LMM models found no differences in strongyles egg CPGs (Table 4-2 and Table 4-3) and trematode (*Paramphistomatid*) CPGs (Table 4-4 and Table 4-5) between translocated elephants and local elephants (individual elephants and family groups). There were no blood fluke eggs (e.g. *Bivitellobilharzia nairi*) observed in this study, although there were at least two types of trematode eggs (different sizes) belonging to *Paramphistomatid* found (see images in Appendix 7.5). For ciliate CPGs, the LMM models found no important variables (no different from null model) for both "translocated vs local individuals" and "translocated vs local family groups" comparisons (see Table 7-4 and Table 7-5 in Appendix 7.6 for likelihood-ratio results).

**Table 4-1: Elephants' identity, age group, status, study sites, sampling timeline, and ciliate CPGs, strongyles egg CPGs and trematode egg CPGs.**

| No. | Elephant Names | Age group | Status | Study sites | Date of collared | Time since release (Days) | No of samples collected (No. of *QC samples) | Detection of ciliates (*QC samples) [Min;Median;Max CPG] | Detection of strongyles (*QC samples) [Min;Median;Max egg CPG] | Detection of trematode eggs (*QC samples) [Min;Median;Max egg CPG] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | Awang Banun | Subadult | Local Individual, Male | Belum-Temengor | 20 Feb 2013 | 62-467 | 16 (11) | 100% [39; 1678; 4691] | 100% [1156; 5234; 11967] | 100% [211; 391; 1194] |
| 2. | Awang Mendelum | Adult | Local Individual, Male | Belum-Temengor | 3 Apr 2013 | 62-320 | 15 (9) | 100% [1685; 8247; 10384] | 100% [2448; 3619; 6816] | 100% [120; 446; 699] |
| 3. | Awang S.Kedah | Subadult | Local Individual, Male | Belum-Temengor | 19 Feb 2013 | 73-536 | 25 (11) | 100% [1715; 4403; 19515] | 100% [530; 1997; 5730] | 100% [145; 585; 956] |
| 4. | Puteri Rafflesia | Adult | Local Individual, Female | Belum-Temengor | 19 Jan 2014 | 0-450 | 16 (11) | 100% [231; 1307; 14306] | 100% [205; 941; 3243] | 87.5% (81.81% ) [0; 220; 1766] |
| 5. | Puah | Family | Local Family | Kenyir | 14 April 2014 | 66-378 | 27 (27) | 100% [563; 3784; 24645] | 100% [206; 1798; 8318] | 100% [197; 788; 2049] |
| 6. | Sireh | Family | Local Family | Kenyir | 13 May 2014 | 42-321 | 33 (27) | 100% [555; 3574; 40110] | 100% [159; 1621; 4296] | 100% [187; 512; 1143] |
| 7. | Sulaing | Family | Local Family | Kenyir | 14 Oct 2014 | 14-192 | 12 (12) | 100% [617; 3087; 25372] | 100% [174; 583; 1569] | 100% [25; 153; 429] |
| 8. | **Awang Halim** | Subadult | Translocated, Male | Belum-Temengor | 20 May 2013 | 0-380 | 22 (11) | 100% [233; 6482; 18064] | 100% [133; 1730; 7097] | 100% [197; 504; 847] |
| 9. | **Awang Tahan** | Adult | Translocated, Male | Kenyir | 27 Oct 2013 | 0-502 | 14 (8) | 100% [125; 3074; 20291] | 85.71% (75.00%) [0; 744; 1389] | 92.86% (87.50%) [0; 204; 1146] |
| 10. | **Awang Badur** | SubAdult | Translocated, Male | Kenyir | 3 Sep 2014 | 0-244 | 16 (15) | 100% [25; 2712; 20553] | 81.25% (86.67%) [0; 86; 273] | 100% [158; 711; 971] |
| 11. | **Awang Bakti** | Subadult | Translocated, Male | Kenyir | 17 Oct 2014 | 0-202 | 12 (9) | 91.67% (88.89%) [0; 2252; 50607] | 100% [252; 1422; 3547] | 83.33% (77.78%) [0; 49; 2571] |
| 12. | **Awang Teladas** | Adult | Translocated, Male | Kenyir | 22 Oct 2014 | 0-186 | 12 (10) | 100% [1561; 4688; 13868] | 100% [92; 278; 1385] | 100% [69; 693; 1857] |
| | | | | | | | | 100% (all elephants) | 100% (all elephants) | 100% (all elephants) |

***** *QC – quality checked for translocated and local individuals (preserved below 8 hours after defecation and high identity confidence.*

#### 4.5.1.1 Further results for strongyles eggs (Count Per Gram)

The LMM comparison for "translocated vs local individuals" (Table 4-2) and "translocated vs local family groups" (Table 4-3) both shared similar results. For strongyles CPGs, 'time since release' (t=2.537, df=43.38, *P*=0.016), 'fGCM linear' (t=-3.057, df=42.56, *P*=0.004) and 'fGCM quadratic' (t=3.405, df=42.62, *P*=0.001) were important for the translocated elephants but not for local individual elephants (see Figure 4-1). Overall, the strongyles egg CPGs had significant negative relationship with the trematodes (*Paramphistomatid*) egg CPGs (t=-2.648, df=47.06, *P*=0.011). The likelihood-ratio selection of important variables for strongyles egg CPGs, see Table 7-4 and Table 7-5 in Appendix.

**Table 4-2: LMM (lmerTest) results for strongyles CPGs translocated vs. local individual elephants**

| Type | Factor | Estimate | Coefficient (S.E.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept (local individuals) | 5.74 | 1.22 | 48.68 | 4.698 | 3.43 – 7.96 | 2.18e-05* |
| | log10(Trematode CPG) | -0.40 | 0.15 | 47.06 | -2.648 | -0.68 – -0.09 | 0.011* |
| | Time since release | -0.001 | 0.001 | 42.31 | -1.881 | -0.003 – 0.00002 | 0.067 |
| | fGCM (linear) | -0.18 | 0.16 | 43.22 | -1.078 | -0.48 – 0.01 | 0.287 |
| | fGCM (quadratic) | 0.01 | 0.01 | 42.78 | 1.119 | -0.01 – 0.02 | 0.269 |
| | Translocated | 3.54 | 2.13 | 46.14 | 1.664 | -0.34 – 7.58 | 0.103 |
| | Translocated*(Time since release) | 0.004 | 0.001 | 43.38 | 2.537 | 0.001 – 0.006 | 0.014* |
| | Translocated*(fGCM, linear) | -1.36 | 0.45 | 42.56 | -3.057 | -2.23 – -0.06 | 0.003* |
| | Translocated*(fGCM, quadratic) | 0.08 | 0.02 | 42.62 | 3.405 | 0.04 – 0.01 | 0.001* |
| Random | Factor | Variance | Std. Dev | CI | | | |
| Elephants' identity | Intercept | 0.44 | 0.67 | 0.35-1.04 | | | |
| Residual | | 0.13 | 0.36 | 0.27-0.41 | | | |

**Table 4-3: LMM (lmerTest) results for strongyles CPGs translocated elephants vs. local family groups**

| Type | Factor | Estimate | Coefficient (S.E.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept (local individuals) | 5.74 | 0.88 | 74.09 | 6.524 | 3.99-7.51 | 7.44e-09* |
| | log10(Trematode CPG) | -0.32 | 0.13 | 79.28 | -2.569 | -0.58 – -0.06 | 0.012* |
| | Time since release | -0.003 | 0.001 | 71.30 | -4.032 | -0.004 – -0.001 | 0.0001* |
| | fGCM (linear) | -0.24 | 0.12 | 71.95 | -1.92 | -0.48 – 0.01 | 0.06 |
| | fGCM (quadratic) | 0.01 | 0.006 | 71.79 | 1.825 | -0.001 – 0.023 | 0.07 |
| | Translocated | 3.46 | 1.96 | 78.16 | 1.765 | -0.44 – 7.38 | 0.081 |
| | Translocated*(Time since release) | 0.004 | 0.001 | 74.94 | 3.808 | 0.002-0.007 | 0.0003* |
| | Translocated*(fGCM, linear) | -1.35 | 0.42 | 72.76 | -3.188 | -2.20 – -0.51 | 0.002* |
| | Translocated*(fGCM, quadratic) | 0.08 | 0.02 | 73.02 | 3.513 | 0.04 – 0.13 | 0.0008* |
| Random | Factor | Variance | Std. Dev | CI | | | |
| Elephants' identity | Intercept | 0.32 | 0.57 | 0.33-1.07 | | | |
| Residual | | 0.11 | 0.33 | 0.28-0.39 | | | |

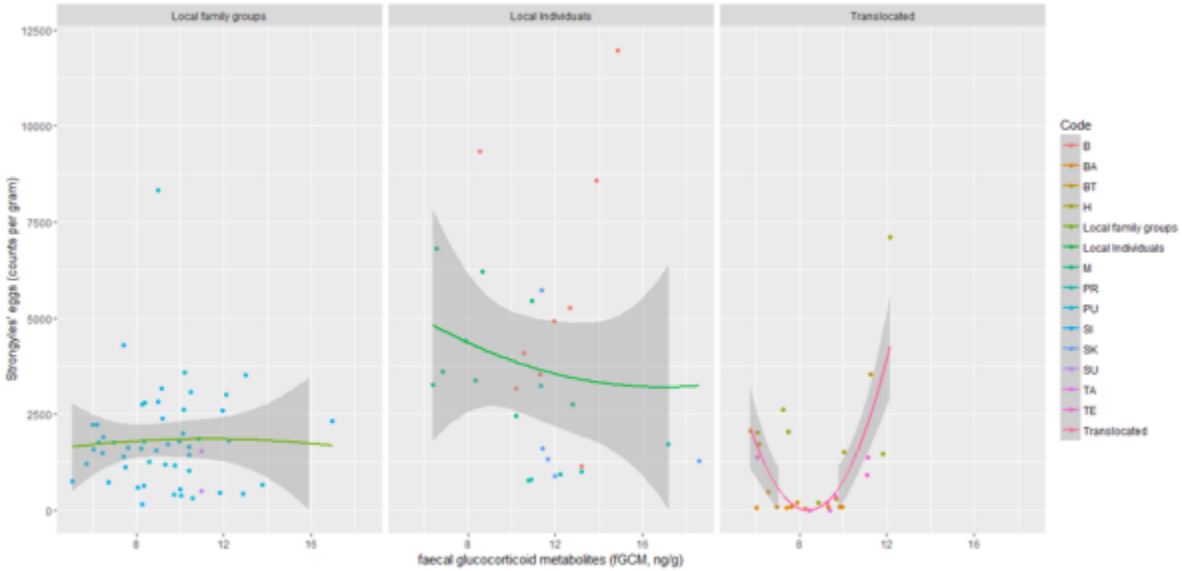


**Figure 4-1: Relationship between faecal glucocorticoid metabolites (fGCM) and strongyles CPGs (Day-60 to Day-365)**

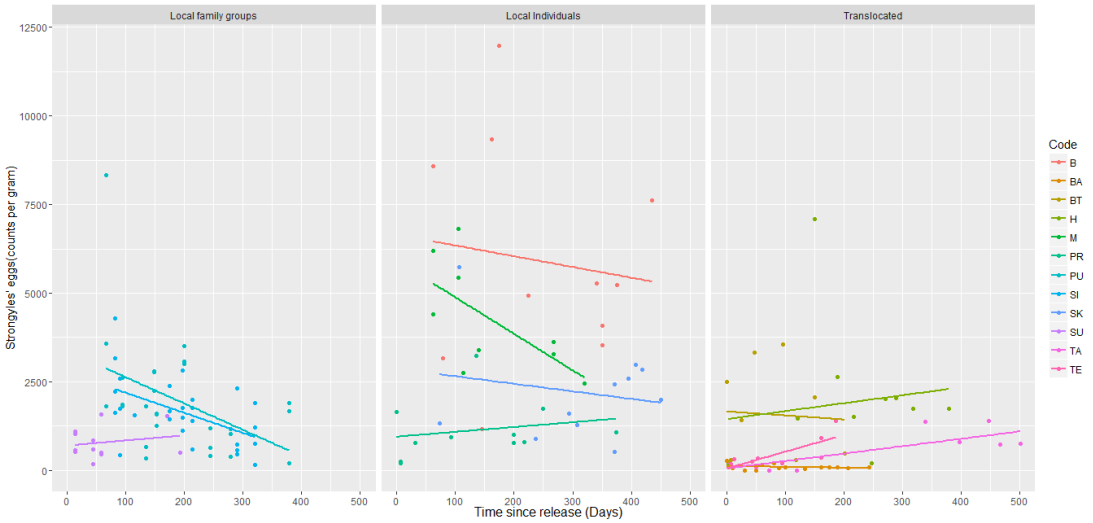


**Figure 4-2: Relationship between strongles egg CPGs and time since release (Day 0 to Day-502)**

### 4.5.1.2 Further results for trematode (*Paramphistomatid*) eggs (Count Per Gram)

When comparing trematode (*Paramphistomatid)* CPGs for translocated and local individual elephants (Table 4-4), the LMM found ciliate CPGs to have positive effect (df=47.16, t=2.28, P=0.027, see Figure 4-3), while strongyles CPGs (df=51.26, t=-4.40, P<0.001) and 'time since release' (df=45.88, t=-3.79, P=0.0004) had negative effect. See Table 7-4 (in Appendix) for likelihood-ratio selection of important variables for the LMM.

The comparison of trematode (*Paramphistomatid)* CPGs in translocated elephants with local family groups (Table 4-5 and Table 7-5) found similar results except ciliates CPGs was not an important variable. In addition, fGCM (linear and quadratic) were identify as important variables for local family groups [fGCM (linear): df=68.08, t=-2.25, *P*=0.028; fGCM (quadratic): df=67.97, t=2.107, *P*=0.039], but not for translocated elephants.

**Table 4-4: LMM (lmerTest) results for trematode CPGs translocated vs. local individual elephants**

| Type | Factor | Estimate | Coefficient (S.E.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept | 3.09 | 0.47 | 51.53 | 6.547 | 2.17 – 4.00 | 2.71e-08* |
| | log10 (strongyles CPG) | -0.36 | 0.09 | 53.08 | -4.219 | -0.53 – -0.17 | 9.63e-05* |
| | log10 (ciliates CPG) | 0.22 | 0.10 | 46.81 | 2.193 | 0.02 – 0.40 | 0.033* |
| | Time since release | -0.002 | 0.0005 | 45.24 | -3.779 | -0.003 – -0.001 | 0.0005* |
| Random | Factor | Variance | Std. Dev | 95% CI | | | |
| Elephants' identity | Intercept | 0.38 | 0.62 | 0.35-1.04 | | | |
| Residual | | 0.09 | 0.30 | 0.24-0.36 | | | |

**Table 4-5: LMM (lmerTest) results for trematode CPGs translocated elephants vs. local family groups**

| Type | Factor | Estimate | Coefficient (S.E.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept | 5.17 | 0.74 | 56.42 | 6.987 | 3.75 – 6.53 | 3.51e-09 |
| | log10(Strongyles CPG) | -0.29 | 0.09 | 73.14 | -3.451 | -0.45 – -0.11 | 0.001* |
| | Time since release | -0.0016 | 0.0004 | 68.51 | -3.370 | -0.002 – -0.001 | 0.001* |
| | fGCM (linear) | -0.24 | 0.11 | 68.08 | -2.251 | -0.447 – -0.035 | 0.028* |
| | fGCM (quadratic) | 0.011 | 0.005 | 67.97 | 2.107 | 0.001 – 0.021 | 0.039* |
| | Translocated | -3.12 | 1.64 | 73.98 | -1.909 | -6.33 – -0.09 | 0.060 |
| | Translocated*(fGCM, linear) | 0.54 | 0.37 | 68.54 | 1.449 | -0.15 – 1.28 | 0.152 |
| | Translocated*(fGCM, quadratic) | -0.03 | 0.02 | 68.4 | -1.25 | -0.068 – 0.013 | 0.214 |
| Random | Factor | Variance | Std. Dev | CI | | | |
| Elephants' identity | Intercept | 0.33 | 0.57 | 0.284 – 0.905 | | | |
| Residual | | 0.09 | 0.29 | 0.242 – 0.336 | | | |

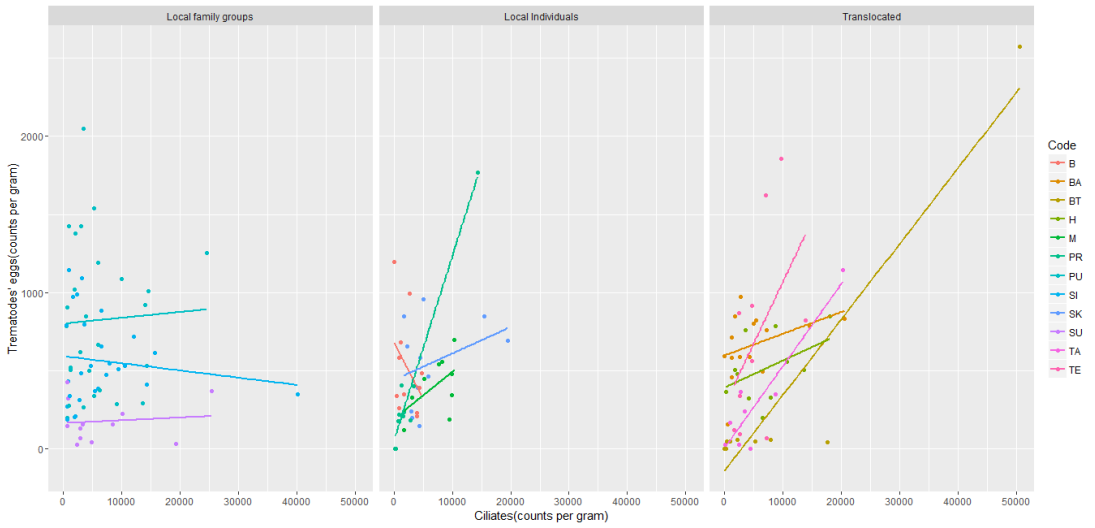


**Figure 4-3: Relationship between trematode (Paramphistomatid) CPGs count and ciliates CPGs (Day 0 to Day-502)**

### 4.5.2 Comparing parasite eggs and ciliates (Count Per Gram) among translocated elephants

In this section, we tried to identify changes over time (categorical, N=5) in parasite eggs and ciliates CPGs for translocated elephants (see LMM results in Table 4-6, Table 4-7 and Table 4-8). The strongyles egg CPGs in translocated elephants was influenced by 'time since release (categorical)' ($\chi^2$=17.551, *P*=0.002, see Figure 4-2). In comparison to the first three months post-release, translocated elephants had a significant increase of strongyles egg CPG counts by end of the first year post-release: Day 271-365 (df=44.88, t=3.159, *P*=0.003) and Day 366-502 (df=44.89, t=3.618, *P*<0.001) (Table 4-6). See Table 7-6 (Appendix) for likelihood ratio results. In contrary to earlier comparison with the local elephants, the LMM did not find fGCM

(linear and quadratic) as significant influence on translocated elephants' strongyles CPGs.

Ciliate counts in dung were generally low (zero counts) during translocation and immediately after release, except for one elephant (BT) that had high ciliate counts in fresh dung collected on the day of translocation (see Appendix, Figure 7-9). Ciliate CPGs were not influenced by time since release (Table 4-7), instead, ciliates CPGs were higher when dung boli were larger (df=35.1, t=2.19, *P*=0.036) and when trematode (*Paramphistomatid*) egg CPGs increases (df=28.53, t=3.324, *P*=0.002). The ciliate CPGs decreases when fGCM (linear) concentrations increases (df=46.76, t=-2.89, *P*=0.006). This result is different to previous LMM results that compared ciliates CPGs between translocated elephants and local elephants, and found no important variable.

For trematode (*Paramphistomatid*) CPGs, two translocated elephants' (see Appendix, Figure 7-9) showed sudden changes in egg counts. One elephant (Bakti (BT)), had a high number of trematode (*Paramphistomatid*) CPGs in the dung collected on the day of translocation, but after release, the numbers had declined and remained low throughout the rest of the sampling period. By contrast, the other elephant (Teladas (TE)) showed progressively increasing trematode (*Paramphistomatid*) CPGs over time. Overall, the LMM found trematode (*Paramphistomatid*) CPGs to be significantly lower at last quarter of the year post-release (Day 271-365), in comparison to the first three months after release (see Table 4-8). The trematode CPGs were positively influenced by ciliates CPGs (df=41.15, t=2.19, *P*=0.034) and average bolus circumference (df=42.89, t=2.094, *P*=0.042).

**Table 4-6: LMM (lmerTest) results for strongyles CPGs among translocated elephants (Day-0 to Day-502)**

| Type | Factor | Estimate | Coefficient (s.e.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept (T1 Day 0-90) | 1.42 | 0.52 | 22.95 | 2.745 | 0.45 – 2.38 | 0.011* |
| | fGCM (linear) | 0.06 | 0.04 | 44.11 | 1.553 | -0.01 – 0.13 | 0.128 |
| | T2 Day 91-180 (vs. T1) | 0.33 | 0.24 | 40.99 | 1.402 | -0.11 – 0.78 | 0.168 |
| | T3 Day 181-270 (vs. T1) | 0.53 | 0.29 | 42.15 | 1.852 | -0.01 – 1.08 | 0.071 |
| | T4 Day 271-365 (vs. T1) | 1.46 | 0.46 | 44.88 | 3.159 | 0.58 – 2.33 | 0.003* |
| | T5 Day 366-502 (vs. T1) | 1.43 | 0.40 | 44.89 | 3.618 | 0.61 – 2.17 | 0.001* |
| Random | Factor | Variance | Std. Dev | 95% CI | | | |
| Elephants' identity | Intercept | 0.36 | 0.60 | 0.23 – 1.22 | | | |
| Residual | | 0.43 | 0.66 | 0.51 – 0.77 | | | |

**Table 4-7: LMM (lmerTest) results for ciliates CPGs among translocated elephants (Day-0 to Day-502)**

| Type | Factor | Estimate | Coefficient (s.e.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept | 0.94 | 0.97 | 35.13 | 0.965 | -0.87 – 2.82 | 0.341 |
| | log10(Trematode CPG) | 0.47 | 0.14 | 28.53 | 3.324 | 0.20 – 0.74 | 0.002* |
| | Average boli circumference | 0.05 | 0.02 | 35.19 | 2.185 | 0.005 – 0.091 | 0.035* |
| | fGCM (linear) | -0.08 | 0.03 | 46.76 | -2.888 | -0.11 – -0.02 | 0.006* |
| Random | Factor | Variance | Std. Dev | CI | | | |
| Elephants' identity | Intercept | 0.07 | 0.25 | 0.00 – 0.54 | | | |
| Residual | | 0.34 | 0.59 | 0.47 – 0.71 | | | |

**Table 4-8: LMM (lmerTest) results for trematode CPGs among translocated elephants (Day-0 to Day-502)**

| Type | Factor | Estimate | Coefficient (s.e.) | Df | t-value | 95% CI | *P*-values |
|---|---|---|---|---|---|---|---|
| Fixed | Intercept (T1 Day 0-90) | -0.52 | 0.88 | 43.14 | -0.586 | -2.15 – 1.16 | 0.561 |
| | log10(Ciliates CPG) | 0.23 | 0.11 | 41.15 | 2.188 | 0.04-0.44 | 0.034* |
| | T2 Day 91-180 (vs T1) | 0.16 | 0.17 | 40.26 | 0.946 | -0.16 –0.49 | 0.350 |
| | T3 Day 181-270 (vs T1) | 0.12 | 0.21 | 40.53 | 0.621 | -0.26 –0.52 | 0.538 |
| | T4 Day 271- 365 (vs. T1) | -0.67 | 0.29 | 41.19 | -2.335 | -1.22 – -0.14 | 0.025* |
| | T5 Day 366-502 (vs. T1) | 0.09 | 0.29 | 41.94 | 0.331 | -0.46 – 0.63 | 0.742 |
| | Average boli circumference | 0.04 | 0.02 | 42.89 | 2.094 | 0.004 – 0.084 | 0.042* |
| Random | Factor | Variance | Std. Dev | 95% CI | | | |
| Elephants' identity | Intercept | 0.32 | 0.57 | 0.27-1.12 | | | |
| Residual | | 0.21 | 0.45 | 0.35-0.53 | | | |

## 4.6 Discussion

In this chapter, we combined GPS satellite collaring, faecal endocrinology and faecal parasitology to monitor fGCM and parasites egg counts in translocated and non-translocated wild Asian elephants in Peninsular Malaysia. To the best of my knowledge, this is the first monitoring of fGCM and parasites egg counts in free-ranging Asian elephants in a tropical rainforest.

There was no evidence of a higher parasite load in translocated elephants compared to local elephants. One of the reasons for this result may be that translocated elephants were usually administered anti-parasitic medication (e.g. Doramectin) before being released and this medication may persist in blood plasma up to 35 days or more (Pérez et al., 2002; Toutain et al., 1997). The prepatent period for trematodes in elephants is around eight weeks, it but can prolong up to sixteen weeks under unfavourable conditions (Fowler and Mikota, 2006). For strongyles, there was no information on prepatent period in elephants (Fowler and Mikota, 2006), but in horses, it was estimated to be around six to 12 months (Shite et al., 2015). Anti-parasitic medication may also reduce microflora ciliates in the gut, and some ciliates showed signs of recovery after six weeks or more (Ramanan et al., 2016; Vincent et al., 2012). In this study, ciliates in wild elephants recovered quickly after translocation and the effect of time since release was not important on ciliates CPGs (count per gram).

The effect of anti-parasitic medication and long prepatent period could explain why some (but not all) translocated elephants started with low number of strongyles egg CPGs that eventually re-establish towards the end of the first year post-release monitoring (Figure 4-2). The strongyles CPGs have a quadratic relationship with fGCM but this was only in detected for translocated elephants (compared between Day-61 to 365 post-release). This relationship was not detected in the overall monitoring of translocated elephants (Day-0 to Day-502 post-release), possibly because of the anti-parasitic medication that was administered to some translocated elephants and confounded the results for the first few months. Chapman et al., (2006) found positive correlation between nematode egg counts and fGCM for wild red Colobus monkeys (*Piliocolobus tephrosceles*), but a different study by Clough et al. (2010) on wild red-fronted lemurs (*Eulemur fulvus rufus*) found the opposite results. These two studies tested only linear correlations and did not consider non-linear (polynomial) effects of fGCM on strongyles egg CPG. On the other hand, the effect of glucocorticoids was not apparent in the local elephants for this study. Since the effect of glucocorticoids on strongyles (and the immune system) becomes more apparent only during stressful situations (Segerstrom and Miller, 2004), we speculate that the non-linear effect of glucocorticoids on strongyles CPGs will not be apparent for 'unstressed' animals due to the immune system that is exerting a density-dependent effect on parasites (Paterson and Viney, 2002). Future studies should consider testing not only elevated concentrations of glucocorticoids but also the effect of lowered glucocorticoid concentrations, in relation to stressful events, on gastrointestinal parasite egg counts and health.

In future, there is a possibility to extend this study to measure the effect of testosterone concentrations (Ezenwa et al., 2012; Klein, 2004; Mougeot et al., 2006) and the combination effect of reproductive hormones and glucocorticoids (Muehlenbein, 2006; Muehlenbein and Watts, 2010) on the parasite egg counts. A bigger hurdle is that parasite egg counts may not be the best method to estimate parasite burden (Christensen et al., 1996; Eddi et al., 1997; Nansen and Roepstorff, 1999; Roepstorff et al., 1996), especially for free-ranging wildlife. There are several other factors that may play a stronger role in influencing parasite infection for free-ranging wildlife such social factors (interaction with other elephants) and environment considerations (parasite intermediate life cycles) which will be elaborated on further below, after discussion on ciliates and trematodes.

The ciliate CPGs' in this study were low during translocation (with the exception of the translocated elephant Bakti (BT)) and this often coincided with high fGCM values and smaller dung size produced during this strenuous period. After the elephants were released, ciliate numbers increased quickly while fGCM dropped (negative relationship), and dung bolus size increased to a more expected size for animals of their body size (positive relationship). Therefore, it is not surprising that the effects described above were only detected within translocated elephants (Day-0 to Day-502 post-release) but not when comparing translocated to local elephants after sixty days post-release. Ciliates are known as symbionts in the gastrointestinal track that assist in the digestion of plant materials (Profousová et al., 2011a; Schmidt et al., 2005). So, it could be that diet (such as starch and fiber content) will be more important in influencing ciliate CPGs than the variables examined in this study (Petrželková et al., 2012; Schovancová et al., 2013).

In the case of trematode (*Paramphistomatid*) CPGs, the positive relationship between trematode and ciliate counts was detected in translocated and local individual elephants but not for local family groups (Figure 4-3). The correlation between counts could reflect sample conditions (e.g. some samples with high extraction of parasites, have high extraction of ciliates as well), but if that is the case, we would expect the correlation to be reflected in family group samples as well, which did not happen. Another possibility is that the quantity of ciliates represents the presence of some nutrient in the body with positive influence for reproduction of trematodes. Unfortunately, we did not find other studies on ciliates and trematodes that can help confirm this possibility. It is not certain if this condition would favour strongyles as well, as the recovery of strongyles from anti-parasitic treatment was slow in this study. Only one translocated elephant TE (Teladas) has showed a high increase in trematode (*Paramphistomatid*) CPGs over time, but it was not accompanied by a high elevation of glucocorticoid (Figure 7-9 in Appendix). Without elevation of glucocorticoids, it is not certain if immunosuppression could take place. Although we can't be certain that the increase in trematode (*Paramphistomatid*) egg CPGs is a result of increase in worm burden, but at the very least, it could indicate an increase in adult female parasites (Stear et al., 1995). Meanwhile, other translocated elephants have higher trematode (*Paramphistomatid*) egg CPGs at the start (after release) which indicates that trematodes were less influenced by the administration of anti-parasitic medication during translocation.

In this study, we found the presences of nematode and trematode eggs in all subjects and almost consistently in all dung samples. A study on wild Bornean elephants also

found high prevalence of nematode and trematode eggs in their samples (Hing et al., 2013) but due to differences in sampling techniques, we are unable to compare the results of both studies directly. Hing et al. uses a modified McMaster floatation technique and they are able to detect cestode ova (i.e. Anoplocephala) in their samples, where else this study did not, Currently, there is no evidence that Anoplocephala causes clinical diseases in elephants (Fowler and Mikota, 2006). Both studies did not detect the presences of blood fluke (*Bivitellobilharzia nairi*) eggs.

When studying free-ranging wildlife, there could be other social and environmental factors with stronger effects on parasite burden and parasite egg CPGs, such as the habitat conditions for parasites transmission and how often the translocated elephants encounter other animals (other hosts or intermediate hosts) in the area. Parasite infections occur when the elephant consumes vegetation with the trematode's metacercaria (often found in habitats near water, with snails) or strongyles' larvae (that had crawled from elephant dung to vegetation) (Fowler and Mikota, 2006). Wild elephants in Africa, India or other locations with a pronounced dry season, often have animals crowding around common water holes which facilitates the transmission of parasites (Baines et al., 2015; Loarie et al., 2009; Perera, 2009; Thurber et al., 2011; Vidya and Sukumar, 2002; Watve and Sukumar, 1995). In contrary, Malaysia has a high availability of food and water all year round, which may result in less nutritional stress and less concentration of wildlife in an area. Moreover, the translocated elephants were often by themselves after they were released and this may further minimise their chance of being infected in comparison to local elephants with high interactions. The role that social and environmental (microhabitat) variables play in

influencing parasite infection and parasite egg counts need to be considered in future studies.

## 4.7 Conclusion

Overall, this study was not able to detect downstream effect of potential stress from translocation on immunity (immunosuppression) through the use of parasite egg CPGs and ciliate CPGs. There are a number of possible reasons why this could be the case: (i) small sample size (number of elephants monitored), (ii) some translocated elephants were treated with anti-parasite medications prior to their release, (iii) translocated elephants had lower fGCM instead of elevated fGCM (uncertain if immunosuppression took place), (iv) parasite egg counts were possibly confounded by other social and environment influences and (v) there were individual differences (e.g. increase of trematode egg counts were detected for the elephant 'Teladas', but not for other translocated elephants).

The source of parasite infection and prepatent period may be important considerations when monitoring of parasites in free-ranging wildlife. This study has found some non-linear interactions between parasites egg counts and fGCM, but there is gap in relating these patterns to the animal's health conditions. There have been some exciting developments in this field, as more researchers have started looking at the interlink between microbiome, parasites, immune system, endocrinology and health conditions in humans (Bested et al., 2013; Neuman et al., 2015; Ramanan et al., 2016; Round and Mazmanian, 2009). The future will be to translate these knowledge and

technology to advance non-invasive wildlife health monitoring (Gomez et al., 2015; Neuman et al., 2015).

# 5 General Discussion

This study is the first longitudinal monitoring of faecal endocrinology in a tropical environment that compares translocated and local wild Asian elephants. The fieldwork is of considerable scale, and involves sharing of extensive resources and active collaboration with the DWNP, MEME and Universiti Kebangsaan Malaysia for access to wild elephants. This study is also amongst the first PhD studies funded by MEME (a collaborative effort between DWNP and UNMC) and Yayasan Sime Darby (YSD), as part of their effort to move towards evidence-based management of elephants in Peninsular Malaysia. Chester Zoo, UK has also contributed significantly to this study in terms of training, funding, manpower and technical expertise.

Non-invasive endocrinology testing using faecal samples or another methods (e.g. hair, feathers, scales) is a fast growing research field (Brown, 2000; Ganswindt et al., 2012; Hodges, 2005). The ability to carry out continuous health monitoring on free-ranging wildlife without the need for capture is an attractive prospect for wildlife conservation and management. The continuous advancement in technology has helped to reduce cost as well as in improving the performance of GPS satellite collars (Blake et al., 2001). Meanwhile, the development of enzyme immunoassays that are easier to set-up in research laboratories in comparison to radioimmunoassay, made it more feasible to study faecal endocrinology (Munro and Stabenfeldt, 1984; Wasser et al., 2000; Watson et al., 2013). These factors have enabled this study to monitor the effect of translocation on wild Asian elephants in a tropical rainforests.

In the beginning of this PhD, a physiological validation for faecal glucocorticoid metabolites (fGCM) and enzyme immunoassays was carried out in August 2012

(results are not presented here). A long-acting synthetic ACTH (Novartis Synacthen, 6mg per elephant) was administered via intramuscular injection to stimulate the adrenal glands to increase production of glucocorticoids in four captive Asian elephants (two adult males and two adult females) at the Ayutthaya Elephant Palace and Royal Kraal, Thailand. All dung piles defecated by these elephants were collected for baseline monitoring until four days after the injection. Unfortunately, the results from two different faecal hormone metabolite (fGCM) extractions and field storage methods were not conclusive on whether the ACTH experiment worked. Further arrangements were made to send the frozen back-up faecal samples from Thailand to Chester Zoo's laboratory in the UK, but they did not detect expected changes in fGCM (a baseline fGCM that increased to a peak and later reduce back to baseline).

The failure of the ACTH experiment could be due to: (i) the past trauma history of the captive elephants in the camp (some were known to be elephants with a problematic past), (ii) the HPA axis and/or adrenal glands were compromised (adrenal insufficiency) or/and (iii) there was a bull in musth chained nearby to the elephants in this study. Due to the elephants' past experience, adrenal or HPA exhaustion could be a reason, such as the case of wild Chukar partridges that were unable to mount an appropriate glucocorticoid response to ACTH injection when taken into captivity initially (Dickens et al., 2009b). However, social environment (relationship with other elephants) and habituation to experiment protocols can have strong influence on glucocorticoid baseline and perhaps were underestimated in this experiment (Sapolsky, 1990; Velthuizen, 2008). Nonetheless, Chester Zoo carried out a successful biological validation for the assay (CJM 006, UC Davis, results presented in Watson et al. 2013). In addition, they ran a high performance liquid

chromatography (HPLC) analysis at Thermo-Fisher Scientific laboratory to confirm that the assay has minimal cross-reactivity with other steroid hormone metabolites (pers. comm. Walker).

One of the main challenges for this study was to adapt faecal endocrinology for sampling in the warm and humid tropical climate of Malaysia. One of the gaps that we first identified through literature review was the unknown stability of fGCM within the first few hours after defecation in tropical climate. In January 2013, we designed and carried out a dung decay experiment for which we collected 685 subsamples, from 80 dung piles by ten elephants. we sampled from the same dung pile multiple times, ranging from defecation time to two days after. We did this to determine the length of time in which fGCM were stable in a tropical field environment. Based on my findings, fGCM are stable for about 8 hours after defecation. This experiment showed that it is important for researchers to consider the amount of time elapsed since defecation when carrying out faecal endocrinology monitoring in the field, as after eight hours fGCM concentrations can artificially elevate over time.

Another key finding from this study is that translocated wild elephants had significantly lower concentrations of fGCM in comparison to local individual elephants from two months up to a year after collaring (five translocated vs. four local individual elephants). This was contrary to the initial expectation that there would be elevated fGCM as a sign of chronic stress in the immediate period after release. Instead, we found that high concentrations of fGCM did not persist after the elephants were release in a new habitat, and their fGCM showed an immediate drop within the

first few days after translocation (taking into account the delay in gut transit time). Although lowering of basal glucocorticoids (cortisol or corticosterone) has been detected in translocated wild Chukar partridges (Dickens et al., 2009a), in chronic stress treatment on wild-caught European starlings (Cyr et al., 2007; Cyr and Romero, 2008, 2007) and in temporarily confined wild-caught black rhinocerous (Linklater et al., 2010), it has not been reported in elephants before. The state of lowered fGCM in the translocated elephants in this study may not be permanent, as two translocated elephants had increased fGCM concentrations to a comparable range with the local elephants after one year post-release.

The effect of a prolonged lowering of glucocorticoid concentrations on health, physiology and behaviour in wild elephants is largely unknown. In humans, hypocortisolism (the state of low glucocorticoids) has been identified in those with acute adrenal crisis, Post-Traumatic Stress Syndrome (PTSD), chronic fatigue syndrome, fibromyalgia, rheumatoid arthritis and asthma amongst others (Arlt and Allolio, 2003; Edwards et al., 2011; Heim et al., 2000; Lee and Ho, 2013; Yehuda et al., 1996). There is also evidence that living in conditions of chronic stress (e.g. having financial difficulties, children experiencing parental divorce, functioning as caregivers of dementia patients) may result in reduced adrenal activity and reactivity (Faresjö et al., 2013; Heim et al., 2000; Koss et al., 2015; Kraft and Luecken, 2009; Leggett et al., 2015). Although psychological disorders such as anxiety, depression and PTSD may exist in animals, there are no existing diagnostic tools that can be used for forest elephants with limited direct observation opportunities (Bradshaw et al., 2005; Clinchy et al., 2011; Katz et al., 1981; Korte et al., 2005; Sheriff et al., 2010b; Zoladz et al., 2012). Future research should consider the development of non-invasive

tools to diagnose adrenal insufficiency and PTSD for free-ranging wildlife in the forest.

We have also investigated gastrointestinal parasite egg counts and microflora ciliate counts and found no significant differences between translocated and non-translocated elephants. The results for faecal parasitology maybe confounded by some medical, social and environmental factors, and we was not able to detect signs of compromised immune function. Future studies should continue testing and developing more non-invasive monitoring techniques to measure the downstream effect of stress on health (e.g. adrenal insufficiency, PTSD, allostatic overload).

The main limitations of this study were the small sample size (number of elephants) and the difficulty in obtaining faecal samples within eight hours of defecation. Translocation and collaring sessions were unpredictable as we often failed to collar an elephant. It was also unfortunate that fGCM monitoring was cancelled for four elephants due to GPS collar failures and poaching. There was also a time limit of two years allocated for fieldwork in this research, so there was no time available to collect additional samples. The small sample size, did not allow reliable comparison of location or sex differences for fGCM concentrations, parasite egg counts and ciliate counts. It is difficult to achieve a large sample size when studying an endangered wildlife species, particularly one which happens to be the largest terrestrial animal in the tropical rainforests of Asia. Nonetheless, by using individual repeated measure sampling, we are confident that a low fGCM state did occur in the translocated elephants. The current limitations of this study can be overcome if this research can be continued and expanded through faecal DNA identification (collected in tandem

with hormone samples) to identify individuals and sex –especially those in family groups – to enable longitudinal fGCM comparison within known subjects.

To understand the complexity of production and physiological impact of glucocorticoids, we need to consider the sensitivity of mineralocorticoid and glucocorticoid receptors as well as glucocorticoid resistance (Chrousos and Kino, 2009; Dickens et al., 2009c; Marques et al., 2009). There is also a need to reconcile previous findings with new findings in the field of endocrinology. For example, some studies confirmed that 'free glucocorticoids' are the biologically active component in the body instead of 'total glucocorticoids' in the blood. (the latter included the measurement of both 'free' and inactive glucocorticoids bound to corticosteroid binding globulins and albumin) (Breuner et al., 2013; Delehanty et al., 2015; Sheriff et al., 2010a). Since the discovery, the topic has been debated (Breuner and Orchinik, 2002; Schoech et al., 2013) and studies measuring glucocorticoids using blood samples were encouraged to report both 'free' and 'total' glucocorticoid concentrations. However, it is difficult to interpret past studies which did not distinguish between the two measurements, which may result in limitations in terms of knowledge (eg. measurement of glucocorticoid metabolites in saliva and faeces reflects the 'free' glucocorticoid component (Sheriff et al., 2010a), and may not correlate with measurement of 'total glucocorticoids' in blood).

Another knowledge limitation is that most endocrinology studies emphasise the relationship between stress and the elevation of glucocorticoids, possibly in part due to Hans Selye's legacy. Through the examples given previously, we have highlighted the fact that there are a growing number of studies associating lower glucocorticoid

concentrations with chronic stress, physiological changes in the HPA axis, and lower health quality (Gunnar and Vazquez, 2001; Heim et al., 2000; Raison and Miller, 2003). Considering that it is widely acknowledged that there is an inverted "U" association between glucocorticoid concentrations and physiological functions (Sapolsky, 2015), concentrations of glucocorticoids which are either too high or too low can be disruptive for physiological functions in the brain and body (Busch and Hayward, 2009; Korte et al., 2007; McEwen et al., 2015). My argument is that the connection between low glucocorticoid concentrations and stress is not an anomaly, and should be investigated further.

Not much is known about the negative effects of persistent low glucocorticoids on the health and behaviour of wild elephants. If this condition reflects a more serious condition such as adrenal or HPA exhaustion, medical intervention may be needed to correct the hormone imbalance and prevent the development of other possible psychological or behavioural anomalies such as PTSD or excessive aggressiveness in translocated elephants. Therefore instead of focusing research solely on the health implications of elevated glucocorticoids, once again we reiterate my point, that wildlife researchers need to consider the effect of low glucocorticoids on health and behaviour. Although we have yet to assess the effect of maternal stress on offspring for wild elephants, but studies on humans and other species suggested the mother's glucocorticoids (tested for both elevated and low concentrations) during pregnancy can affect offspring's stress response, behaviour and health (Franklin et al., 2010; Matthews and Phillips, 2012; Sheriff et al., 2010b).

## Conclusion

In terms of human-elephant conflict (HEC) management, conservation authorities and other relevant stakeholders have to consider that the problem does not end at the release site. The results of this study confirmed that translocated elephants had prolonged lower glucocorticoid concentrations that are of health concern. We speculate that these physiological changes in an elephant would result in an altered response to stressors and an inability to react appropriately to danger that will leave the elephant in a vulnerable state (Dickens et al., 2010, 2009a; Koolhaas et al., 1999). In terms of behaviour, they may exhibit extreme behaviours such as increased aggressiveness or avoidance behaviour (Koolhaas et al., 1999; Popma et al., 2007; Terburg et al., 2009).

The assessment of HEC mitigation success needs to include consideration of elephants' health. Although there are always room for more research, the existing results and literature are pointing towards the need to exercise precautionary principles. Translocation is a stressful and disruptive process for wild elephants and may result in social/ society collapse with possible dire consequences to wildlife behaviour and health. Instead, we have to find ways to encourage human and elephant coexistence at local landscapes and other HEC mitigation methods should be considered. In the scenario where translocation is unavoidable, we would also suggest that monitoring for translocated elephants to be carried out for two years or more whenever it is possible.

As natural habitat for wildlife is shrinking due to anthropogenic development, management of free-ranging wildlife is predicted to become more intensive for species conservation (lecture given by 2016 Indianapolis Prize winner, Prof. Carl Jones, Durrel Wildlife Conservation Trust). Monitoring of wildlife physiology can help us understand the impact of anthropogenic disturbances on free-ranging wildlife and conserve a healthy population for long-term conservation success (Cooke et al., 2013; Wikelski and Cooke, 2006). Non-invasive monitoring of endocrine hormones, parasites and gut microbiomes from faecal samples opens up new possibilities for monitoring the health of free-ranging wildlife populations; and efforts should be made to continue developing this area of research. There is also potential to include movement analysis in fGCM analysis to better understand the differences between translocated and local elephants, which we hope to continue in near future.

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# 7 Appendices

## 7.1 Chapter Two: Weather parameters

**Table 7-1: Weather data 13-19 January 2013**

| Date | Sunshine * (hour) | Global Radiation** ($MJm^{-2}$) | Temperature at 8.00am* (° Celsius) | Temperature at 2.00pm* (° Celsius) | 24 hour mean temperature* (° Celsius) | 24 hour mean relative humidity* (%) | Time and duration of rainfall* (minutes) | Amount of rainfall (mm)* |
|---|---|---|---|---|---|---|---|---|
| 13 Jan 2013 | 7.10 | 12.87 | 23.6 | 29.5 | 26.2 | 82.5 | 21:00 (70 min) | 18.8 |
| 14 Jan 2013 | 9.30 | 18.97 | 23.1 | 29.2 | 26.4 | 80.2 | 0 | 0 |
| 15 Jan 2013 | 4.65 | 12.37 | 23.2 | 28.7 | 26.1 | 79.9 | 20:00 (8 min) | 0.1 |
| 16 Jan 2013 | 3.05 | 8.53 | 23.1 | 28.9 | 25.7 | 82.2 | 0 | 0 |
| 17 Jan 2013 | 7.65 | 18.19 | 23.0 | 29.2 | 26.0 | 76.6 | 0 | 0 |
| 18 Jan 2013 | 1.20 | NA | 22.2 | 25.4 | 23.6 | 89.3 | 12:00 (16 min) 13:00 (28min) 15:00 (214min) | 14.7 |
| 19 Jan 2013 | 1.10 | 7.45 | 22.8 | 28.2 | 24.8 | 85.4 | 0 | 0 |

* Weather data obtained from Station Felda PPP Tun Razak (03° 50'N, 102° 34'E, 76m above mean sea level)
** Weather data obtained from Station Muadzam Shah (03° 03'N, 103°05'E, 33.3m above mean sea level)

**(Source: the Department of Meteorology, Malaysia)**

## 7.2 Chapter Two: Subsamples collected for dung decay experiment

**Table 7-2: Number of faecal glucocorticoid metabolites (fGCM) subsamples collected according to each time sector and experiment treatment.**

| **TIMESECTOR** | | **Open** | **Shade** | **Total samples for each time sector** |
|---|---|---|---|---|
| Time 0 (fresh) | Water | 20 | 21 | 80 |
| | Dry | 22 | 17 | |
| After Time 0 to 2 hours | Water | 20 | 23 | 87 |
| | Dry | 26 | 18 | |
| Above 2 hours to 4 hours | Water | 14 | 16 | 62 |
| | Dry | 19 | 13 | |
| Above 4 hours to 6 hours | Water | 14 | 14 | 58 |
| | Dry | 13 | 17 | |
| Above 6 hours to 8 hours | Water | 14 | 18 | 61 |
| | Dry | 19 | 10 | |
| Above 8 hours to 11 hours | Water | 14 | 14 | 63 |
| | Dry | 22 | 13 | |
| Above 11 hours to 16 hours | Water | 13 | 20 | 60 |
| | Dry | 16 | 11 | |
| 1 Day | Water | 21 | 19 | 78 |
| | Dry | 21 | 17 | |
| 1.5 Days | Water | 17 | 15 | 69 |
| | Dry | 20 | 17 | |
| 2 Days | Water | 18 | 16 | 72 |
| | Dry | 21 | 17 | |
| **Total** | | | | 690 |

## 7.3 Chapter Two: Checking final LMM's model assumption of homogeneity and normality

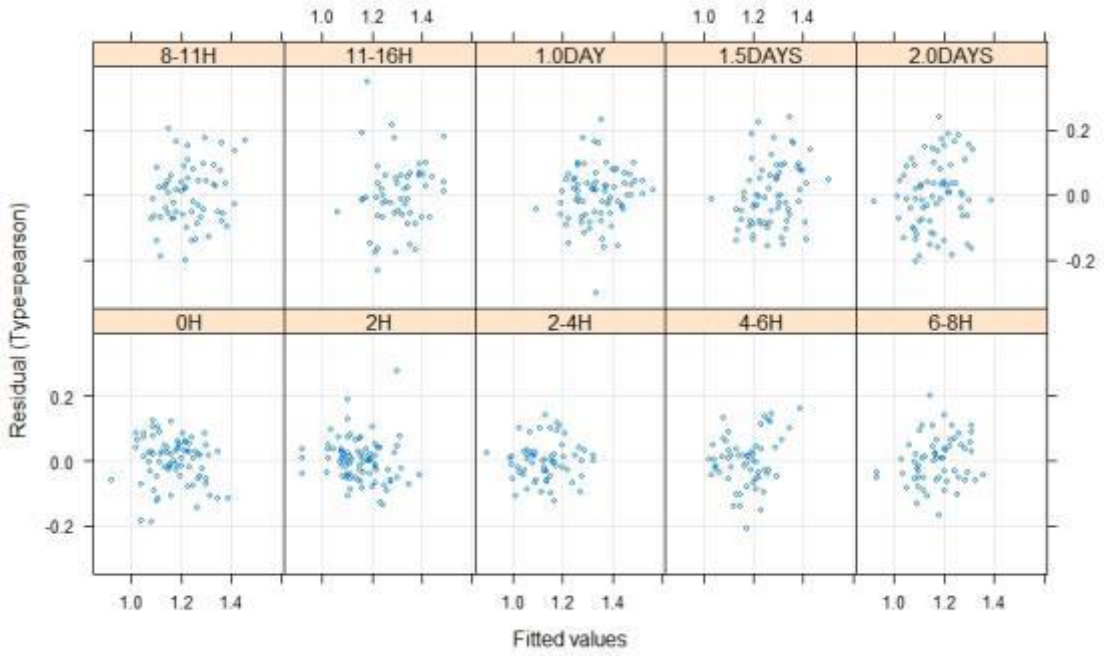


**Figure 7-1: Final LMM's residuals versus fitted values (homogeneity test) according to different time sectors.**

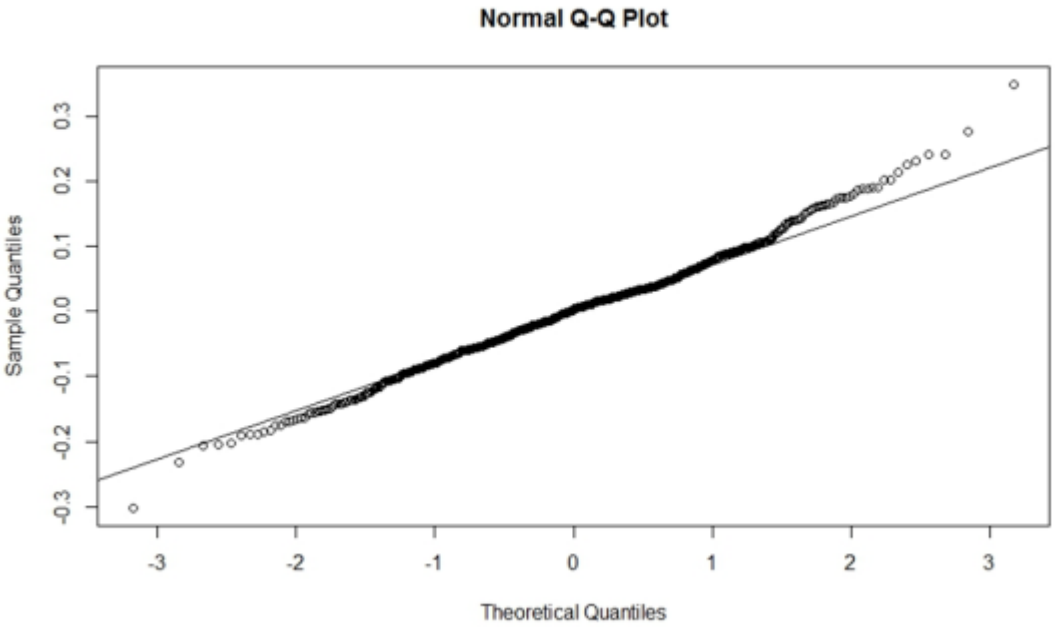


**Figure 7-2: QQ-Plot for final LMM's residuals (normality test)**

## 7.4 Chapter Three: Additional information on medication and supplements administered to translocated elephants

**Table 7-3: Medication and supplements administered to translocated elephants by veterinarians on-site.**

| Medicine type | Medication, dosage and brand | Additional information |
|---|---|---|
| Antihelmintic/ antiparasitic injection (intramuscular/ subcutaneous) | Doramectin (0.02mg/kilogram body weight) - Dectomax® | Given when there are presence open/maggot wound and veterinarian supervision |
| Topical creams for open wound | Acriflavine/ iodine (antimicrobial cream)<br><br>Coumaphos (fly repellent) - Negasunt powder®<br><br>Chlortetracycline/sulfanilamide (antibiotic spray) - Orospray® | |
| Antibiotic injections (intramuscular/ subcutaneous) | Procaine Penicillin - BenacillinR, long acting 3-5 days | Treatment of pus/purulent wound. |
| Supplement injection: (intramuscular/ subcutaneous) | Adult 20-40 ml; Subadult/juvenile/baby 10-20 ml.<br><br>Multivitamin (e.g. vit A, D, B-complex, iron) + amino acid: Vitavet®/Fercobsang®/Biocatalin ®/Catosal® | Given on supplementary basis depending on condition of animal and veterinarian supervision. |

## 7.5 Chapter Four: Gastrointestinal parasite eggs and gut microflora ciliates found in wild Asian elephant dung.


**Figure 7-3: Gastrointestinal nematode (strongyles) parasite egg.**

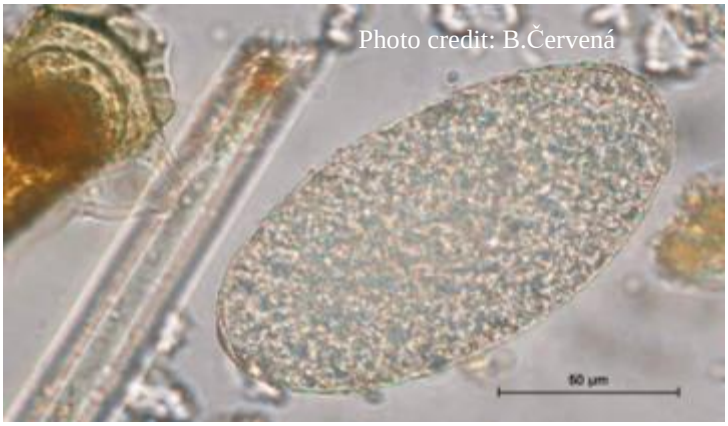


**Figure 7-4: Trematode *Paramphistomatid* egg (Type 1)**

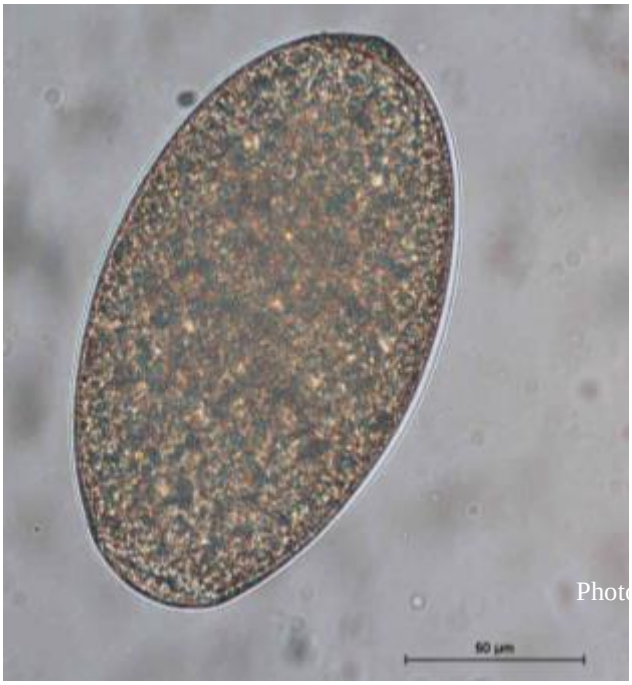


**Figure 7-5: Trematode *Paramphistomatid* egg (Type 2)**

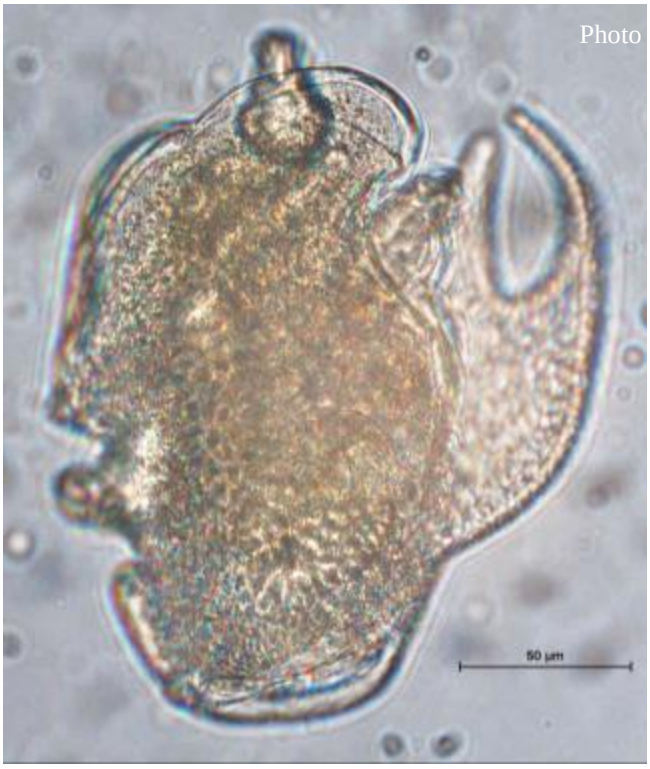


**Figure 7-6: Microflora ciliate (Sample 1)**


**Figure 7-7: Microflora ciliate (Sample 2)**

**Figure 7-8: Microflora ciliate (Sample 3)**

## 7.6 Chapter Four: Likelihood ratio results for parasite egg counts and ciliate counts between translocated and local individual elephants.

**Table 7-4:Likelihood-ratio (LMM) results for parasites egg CPGs and ciliates CPGs between translocated and local individual elephants.**

| TRANSLOCATED VS. LOCAL INDIVIDUAL ELEPHANTS (Day-61 to Day-365) | | | | |
|---|---|---|---|---|
| **Dependent variable** | **Term** | **Df** | **Likelihood-Ratio** | |
| | | | **$\chi^2$-value** | **P-value** |
| **log10(Strongyles CPG+1)** | ***Status × fGCM (linear and quadratic)*** | ***2*** | ***28.318*** | ***7.092e-07*** |
| | ***Duration × Status*** | ***1*** | ***5.2452*** | ***0.0220*** |
| | ***log10(Trematode CPG +1)*** | ***1*** | ***7.695*** | ***0.0055*** |
| | log10(Ciliates CPG + 1) | 1 | 1.2469 | 0.2641 |
| | Average bolus circumference (cm) | 1 | 0.1906 | 0.6624 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.0314 | 0.3098 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 3.5264 | 0.1715 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 2.9911 | 0.2241 |
| | Null Model | 8 | 40.448 | 2.643e-06 |
| **log10(Ciliates CPG+1)** | ***Null Model*** | ***1*** | ***1.6211*** | ***0.2029*** |
| | Status × fGCM (linear and quadratic) | 2 | 2.9603 | 0.2276 |
| | Status | 1 | 0.3956 | 0.5294 |
| | fGCM (linear) | 1 | 0.0685 | 0.7936 |
| | fGCM(quadratic) | 1 | 0.0126 | 0.9105 |
| | log10(Trematode CPG +1) | 1 | 3.7023 | 0.0543 |
| | Duration × Status | 1 | 0.8411 | 0.3591 |
| | Duration | 1 | 0.0037 | 0.9516 |
| | log10(Strongyles CPG +1) | 1 | 0.0148 | 0.9033 |
| | Average bolus circumference (cm) | 1 | 2.5158 | 0.1127 |
| | Location (Kenyir or Belum-Temengor) | 1 | 0.1334 | 0.7150 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 4.7835 | 0.0915 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 2.587 | 0.2743 |
| **log10(Trematode CPG+1)** | ***Duration*** | ***1*** | ***11.631*** | ***0.0006*** |
| | ***log10(Ciliates CPG +1)*** | ***1*** | ***3.8724*** | ***0.0490*** |
| | ***log10(Strongyles CPG+1)*** | ***1*** | ***11.676*** | ***0.0006*** |
| | Status × fGCM (linear and quadratic) | 2 | 3.7107 | 0.1564 |
| | fGCM(quadratic) | 1 | 3.1942 | 0.0739 |
| | fGCM (linear) | 1 | 1.8495 | 0.1738 |
| | Status | 1 | 2.4491 | 0.1176 |
| | Duration × Status | 1 | 1.0458 | 0.3065 |
| | Average bolus circumference (cm) | 1 | 0.8236 | 0.3641 |
| | Location (Kenyir or Belum-Temengor) | 1 | 2.1008 | 0.1472 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 2.5314 | 0.2820 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 0.4707 | 0.7904 |
| | Null | 2 | 24.319 | 2.143e-05 |

## 7.7 Chapter Four: Likelihood ratio results for parasite egg counts and ciliate counts between translocated and local family groups.

**Table 7-5: Likelihood-ratio (LMM) results for parasites egg CPGs and ciliates CPGs between translocated and local family groups.**

| TRANSLOCTED VS. LOCAL FAMILY GROUPS (Day 61 to Day-365) | | | | |
|---|---|---|---|---|
| **Dependent variable** | **Term** | **Df** | **Likelihood-Ratio** | |
| | | | **$\chi^2$-value** | **P-value** |
| **log10(Strongyles CPG+1)** | ***Status × fGCM (linear and quadratic)*** | ***2*** | ***14.953*** | ***0.0006*** |
| | ***log10(Trematode CPG+1)*** | ***1*** | ***5.8793*** | ***0.0153*** |
| | ***Duration × Status*** | ***1*** | ***11.796*** | ***0.0006*** |
| | log10(Ciliates CPG+1) | 1 | 0.6946 | 0.4046 |
| | Average bolus circumference (cm) | 1 | 0.6221 | 0.4303 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.1418 | 0.2853 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 1.2394 | 0.5381 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 1.5724 | 0.4556 |
| | Null Model | 8 | 47.892 | 1.036e-07 |
| **log10(Ciliates CPG+1)** | **Null model** | **1** | **1.8913** | **0.1691** |
| | Status × fGCM (linear and quadratic) | 2 | 0.9788 | 0.6130 |
| | Status | 1 | 1.7802 | 0.1821 |
| | fGCM (linear) | 1 | 0.2918 | 0.5891 |
| | fGCM(quadratic) | 1 | 0.771 | 0.3799 |
| | log10(Trematode CPG+1) | 1 | 1.5823 | 0.2084 |
| | Duration × Status | 1 | 1.1611 | 0.2812 |
| | Duration | 1 | 1.6546 | 0.1983 |
| | log10(Strongyles CPG+1) | 1 | 0.0335 | 0.8547 |
| | Average bolus circumference (cm) | 1 | 0.0024 | 0.9607 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.9422 | 0.1634 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 0.5766 | 0.7495 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 4.1502 | 0.1255 |
| **log10(Trematode CPG+1)** | ***Status × fGCM (linear and quadratic)*** | ***2*** | ***6.614*** | ***0.0366*** |
| | ***Duration*** | ***1*** | ***8.8386*** | ***0.0029*** |
| | ***log10(Strongyles CPG+1)*** | ***1*** | ***10.545*** | ***0.0012*** |
| | log10(Ciliates CPG+1) | 1 | 2.998 | 0.0834 |
| | Duration × Status | 1 | 1.7704 | 0.1833 |
| | Average bolus circumference (cm) | 1 | 1.3562 | 0.2442 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.9005 | 0.1680 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 0.9831 | 0.6117 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 0.7167 | 0.6988 |
| | Null | 7 | 29.962 | 9.65e-05 |

## 7.8 Chapter Four: Likelihood ratio results for parasite egg counts and ciliate counts within translocated elephants.

**Table 7-6: Likelihood-ratio (LMM) results for parasites egg CPGs and ciliates CPGs among translocated elephants.**

| **WITHIN TRANSLOCATED ELEPHANTS (Day-0 to Day-502)** | | | | |
|---|---|---|---|---|
| **Dependent variable** | **Term** | **Df** | **Likelihood-Ratio** | |
| | | | **$\chi^2$-value** | **P-value** |
| **log10 (Strongyles CPG+1)** | ***Duration (TIMESECTOR)*** | ***1*** | ***17.551*** | ***0.0015*** |
| | ***fGCM (linear)*** | ***1*** | ***5.0853*** | ***0.0241*** |
| | Average bolus circumference (cm) | 1 | 2.5516 | 0.1102 |
| | log10(Ciliates CPG+1) | 1 | 0.0000 | 0.9979 |
| | fGCM (quadratic) | 2 | 0.0625 | 0.8026 |
| | log10 (Trematode CPG+1) | 1 | 2.7258 | 0.0987 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.3901 | 0.2384 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 3.5183 | 0.1722 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 2.7011 | 0.2591 |
| | Null Model | 6 | 16.779 | 0.0049 |
| **log10(Ciliates CPG+1)** | ***fGCM (linear)*** | ***1*** | ***13.362*** | ***5.232e-05*** |
| | ***log10(Trematode CPG+1)*** | ***1*** | ***10.92*** | ***0.00095*** |
| | ***Average bolus circumference (cm)*** | ***1*** | ***5.4428*** | ***0.0197*** |
| | fGCM(quadratic) | 1 | 0.2031 | 0.6522 |
| | Duration (TIMESECTOR) | 4 | 4.4500 | 0.3485 |
| | log10(Strongyles CPG+1) | 1 | 0.7990 | 0.3714 |
| | Location (Kenyir or Belum-Temengor) | 1 | 0.2996 | 0.5842 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 1.1241 | 0.5700 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 1.706 | 0.4261 |
| | Null model | 3 | 25.249 | 1.369-e05 |
| **log10 (Trematode CPG+1)** | ***Duration (TIMESECTOR)*** | ***1*** | ***9.7796*** | ***0.0443*** |
| | ***Average bolus circumference (cm)*** | ***1*** | ***4.2412*** | ***0.0395*** |
| | ***log10(Ciliates CPG+1)*** | ***1*** | ***5.2929*** | ***0.0214*** |
| | fGCM (quadratic) | 2 | 2.8059 | 0.0939 |
| | fGCM (linear) | 1 | 1.0616 | 0.3028 |
| | log10 (Strongyles CPG+1) | 1 | 1.1274 | 0.2883 |
| | Location (Kenyir or Belum-Temengor) | 1 | 1.2255 | 0.2683 |
| | Microhabitat 1 (sun, shade, unknown) | 2 | 4.7848 | 0.0914 |
| | Microhabitat 2 (on soil, vegetation, unknown) | 2 | 2.7043 | 0.2587 |
| | Null Model | 6 | 25.849 | 0.0002 |

## 7.9 Chapter Four: Faecal glucocorticoid metabolites, gastrointestinal parasite eggs and ciliates (Count Per Gram) profiles for translocated elephants, local individuals and local family groups.

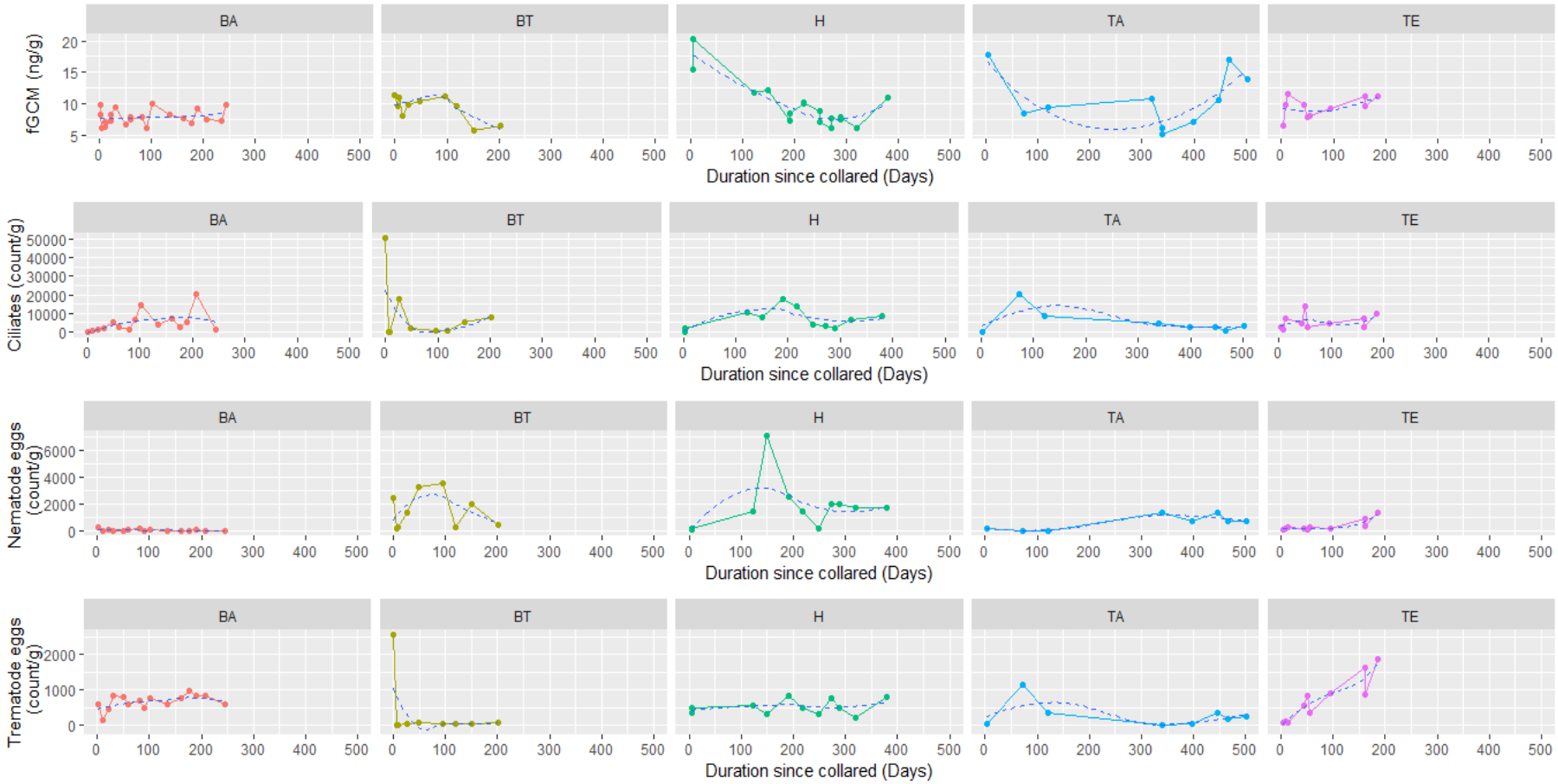


**Figure 7-9: Faecal glucocorticoid metabolites (fGCM), gastrointestinal nematode (strongyles) CPGs, trematodes (*Paramphistomatid*) CPGs and ciliates CPGs for translocated elephants.**

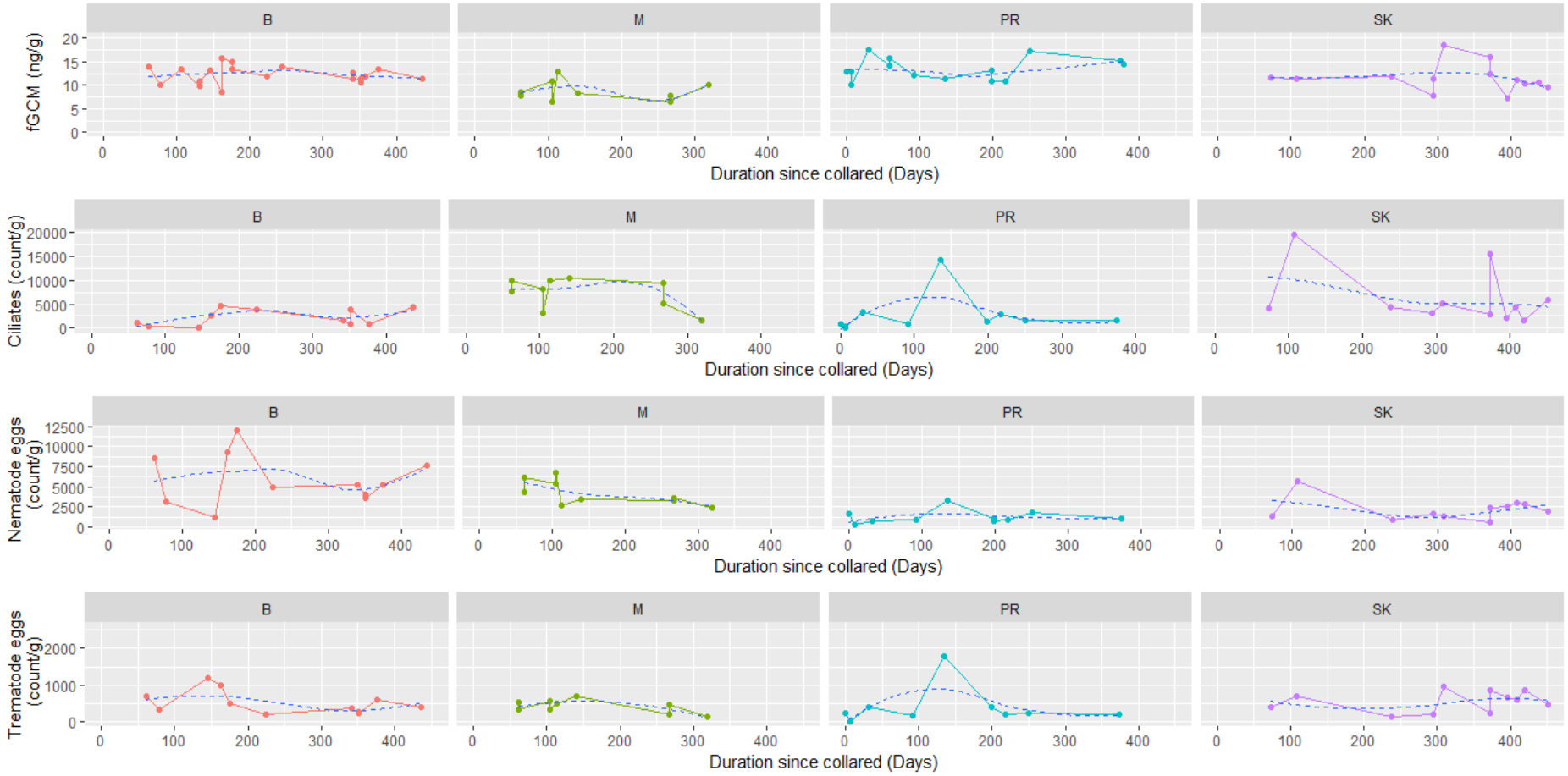


**Figure 7-10: Faecal glucocorticoid metabolites (fGCM), gastrointestinal nematode (strongyles) CPGs, trematodes (*Paramphistomatid*) CPGs and ciliates CPGs for local individual elephants**

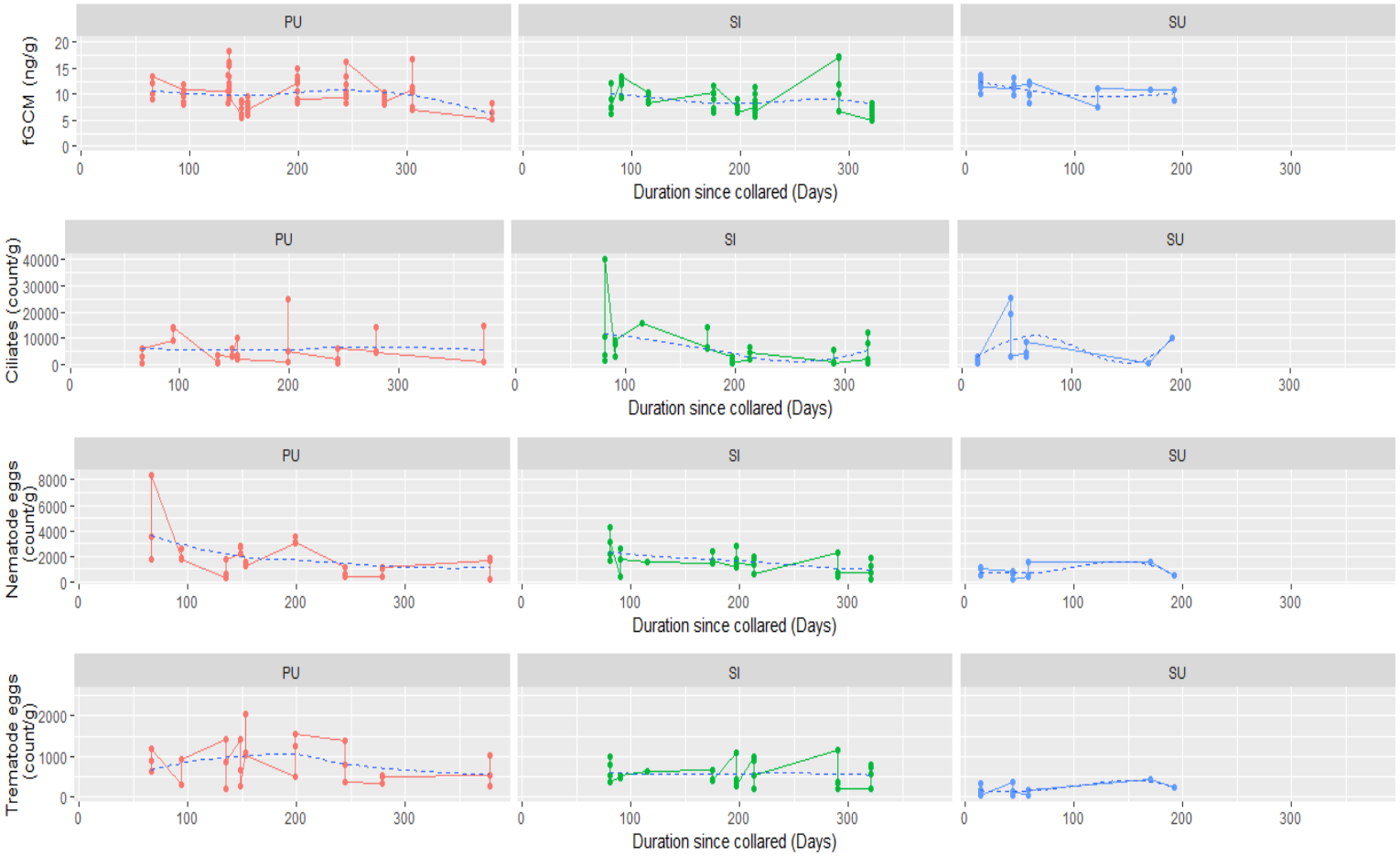


**Figure 7-11: Faecal glucocorticoid metabolites (fGCM), gastrointestinal nematode (strongyles) CPGs, trematodes (*Paramphistomatid*) CPGs and ciliates CPGs for local elephant family groups.**

## 7.10 Photos from fieldwork

**Figure 7-12: Awang Badur was captured near a village and was secured by chains to a tree.**

**Figure 7-13: Two trained captive elephants were brought in from Kuala Gandah National Elephant Conservation Center (NECC) to pull out Awang Badur from the captured site.**

**Figure 7-14: The translocation operation was carried out by En. Nasharuddin Othman and his team from NECC, and the State Department of Wildlife and National Parks (DWNP) of Terengganu.**

**Figure 7-15: Awang Badur was loaded on to a truck to be transported to the National Park.**


**Figure 7-16: The GPS satellite collar**

**Figure 7-17: Awang Badur was collared with the GPS satellite collar in the evening during rain.**

**Figure 7-18: Sighting of Awang Badur a few days after release.**

**Figure 7-19: Locating a dung pile from Awang Badur together with the field tracking team.**

**Figure 7-20: A local resident elephant at Belum-Temengor Forest Complex, Awang Mendelum (walking in front) together with another male elephant.**


**Figure 7-21: Jalong (a translocated female elephant) and her newborn baby.**