Project title: The use of C-reactive proteins as an early disease indicator in captive seals.

Aims and significance

The main aims of this project are to:

1. Initially validate the suitability of commercially available ELISA kits for C-reactive proteins for serum and faecal samples from captive pinnipeds (seals) using the Australian Sea Lion (*Neophoca cinerea*) as the initial species for validation purposes.

2. Once the validation process has been completed a small group of seals will be selected and subjected to varied situations to induce a small level of stress within these animals. This has been found in other species to mimic the reaction that is observed in early disease states and therefore indicate the suitability of these kits to be used in seals to monitor early disease situations (Petersen *et al*., 2004) (see diagram in literature review).

C-reactive protein assays have been used previously in a number of domestic animal species to indicate and monitor disease states but they have not as yet been used in pinnipeds thus, this will be the first use of this type of assay for these species. Furthermore, within the captive environment there is a need to pre-empt the possibility of disease occurrence. This is especially true for those animals involved in performances and being shown to the public. Animals often become stressed and therefore prone to disease. The possibility of being able to predict this in advance is firstly beneficial for the institution involved as preventative measures can be instigated therefore not only alleviating the possible monetary losses due to the need for the care of the animal but also avoiding the negative public perception when they view an animal that is behaving abnormally due to disease. This early detection of disease also has significant benefits for the welfare of the animals because they can be monitored more closely than their counterparts and if needed given preventative care before a more serious disease state results.

More significantly the assay once fully validated could also be used as surveillance for possible disease not only in the captive animals but also could be used in wild populations to monitor their health status. Therefore validation of this test for disease monitoring using faecal samples is highly significant especially for endangered species such as the Australian sea lion

Brief Literature Review

An animal's body has an early defence response to trauma, inflammation, infection or stressors, which is an acute phase response involving a complex set of systemic reactions observed shortly after exposure to a triggering event(see Figure 1, Petersen, *et al*., 2004). One of the many components is an acute phase protein response which causes increased hepatic synthesis leading to increased serum concentrations of positive acute phase proteins. These proteins include C-reactive protein and haptoglobins and such proteins have been used as non-specific indicators of health in large animal veterinary medicine such as the health status of pigs at a herd level, for detection of mastitis in dairy cattle and for the prognosis of respiratory diseases in cattle (Peterson et al., 2004). Furthermore they have provided valuable diagnostic information in the detection, prognosis and monitoring of disease not only in humans but in companion animals and farm herds as well (Eckersal, 2000). These proteins initially released into the blood have been found to be excreted in their pure form in faeces thus measurement of these proteins can easily be done in either sample type. Furthermore, faecal samples have the advantage over the collection of blood in that they do not require the capture and restraint of an animal. It has long been recognised that capture or restraint of animals especially those that are at times fractious increases stress and it is therefore more beneficial where possible to use faecal excretions to monitor these animals as has been shown by Schwarzenberger et al. (1996) for the use of monitoring reproduction in various species.

Commercially available kits using a simple ELISA are available for measurement of concentrations for C-reactive proteins and haptoglobins for a number of species. These use a colorimetric system where a final colour change is directly proportional to the concentration of protein present in the original sample. This colour change for each sample is then measured by a microplate reader at 450nm and converted against a standard to a concentration of the protein within the original sample. The use of C-reactive protein measurement kits is preferable over haptoglobin because haptoglobin is generally a species specific protein unlike C-reactive proteins and also commercially at this stage only available for serum or plasma. The collection of such samples is often difficult in pinnipeds therefore the alternative using C-reactive proteins is preferred as these are less species specific and also measure concentration in a number of body tissues and excreta such as urine and faeces. The collection of faeces from captive pinnipeds, in particular the Australian sea lion (*Neophoca cinerea*) is not difficult and has been done before by the investigator for research during completion of a PhD (Dutton, 2003). This species will again be used for this study as previous connections with the institutions involved will facilitate sample collection.

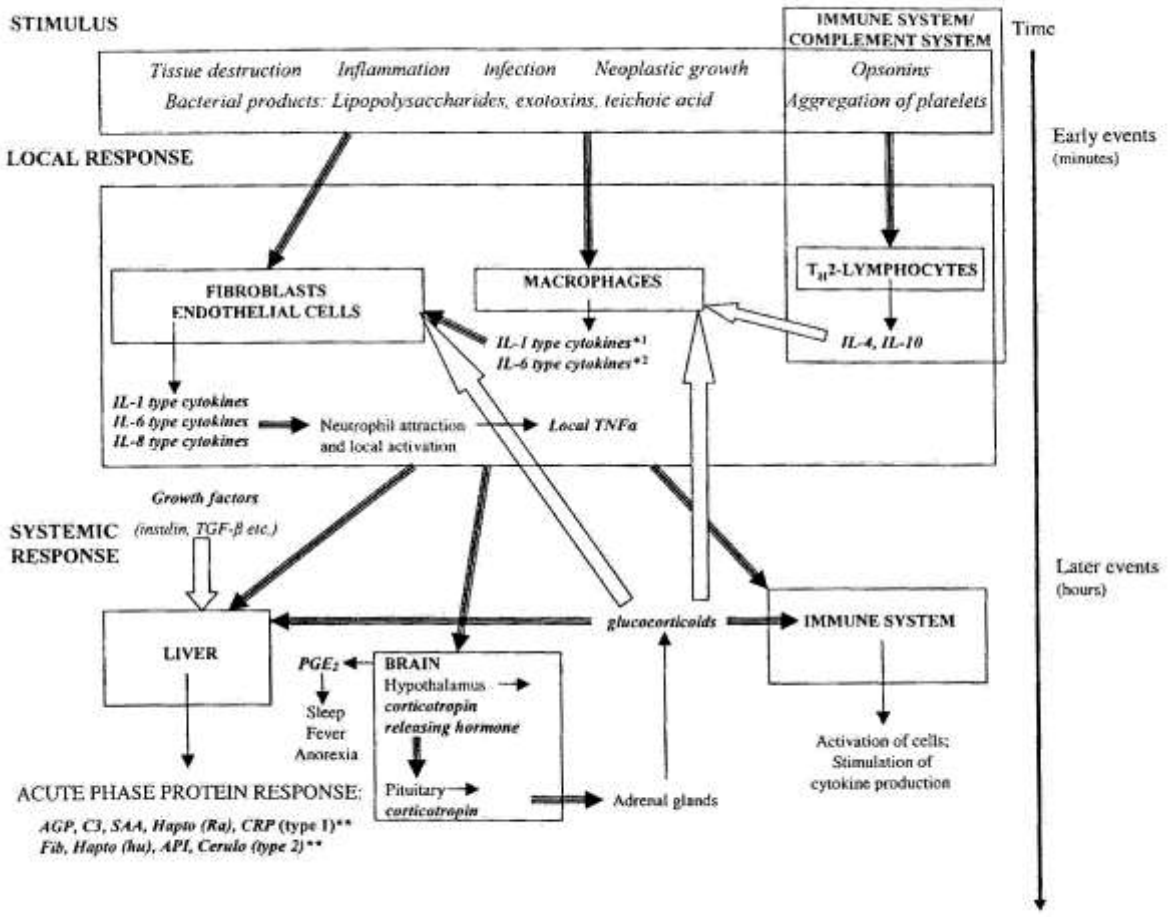

Figure 1: The events involved in the acute phase response in animals (augmented from Petersen *et al.*,2004)

References

Dutton, G (2003). Methods of Assessing Marine Mammal Reproduction PhD Thesis University of Sydney (Unpublished)

Eckersal, PD (2000). Recent advances and future prospects for the use of acute phase proteins and markers of disease in animals. *Revue Med. Vet.* **151(7)**:577-584

Petersen, H.H., Nielsen, J.P. & Heegaard, P.M.H. (2004). Application of acute phase protein measurements in veterinary clinical chemistry. *Vet.Res.* **35**:163-187

Schwarzenberger, F., Möstl, E., Palme, R. and Bamberg, E. (1996). Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Animal Reproduction Science* **42**:515-526

Research Plan, Methodology and Data Analysis

Small groups of Australian sea lions are kept at various institutions in Australia including Pet Porpoise Pool, Coffs Harbour which will be used for initial studies. Sample collection from animals was previously conducted during the principal investigators PhD studies. These animals again will be used for this study as contacts made previously will expedite the sample collection process.

In order to give the best correlation between events and C-reactive proteins there is a need to collect as many samples as possible. It has been found that it is more beneficial to initially get staff at institutions to collect material routinely (even if this becomes daily if samples are available) as the routine of collection will ensure that this is always done. It has been the primary investigators experience that without this routine, sample collection may be forgotten causing vital

periods of data collection to be lost. Samples once collected will be sorted with respect to husbandry events and any disease states that have been noted. Where it is noted that significant influences on health or stress have occurred then these samples will be used for analysis and all others will be kept for possible future use.

Faecal samples are collected from individuals that are routinely housed within individual pens at night. This allows identification of which animal the faeces collected originated from. The samples are collected from the night pen floor during routine cleaning in the mornings when the animals are released to their outdoor enclosure. Therefore the animals are not stressed in this situation as the procedures conducted are normal routine and thus eliminating the stress factor that may occur if the animal were restrained to collect the faecal material. Blood samples will only be collected if this procedure is being conducted for other medical reasons as it is difficult and animals often refuse this procedure if conducted regularly. The procedure though is done for regular health checks and this is when these sample types will be collected. The collected samples will be frozen and stored until bulk shipment to CSU for analysis. This collection will occur over a relatively short period of time, less than one month, as these samples will form the group of samples used for the initial validation process. These initial samples will be subjected to initial assays using the commercial kits and to the initial validation processes as described below:

Intra-assay variation, for this coefficient, five representative aliquots of a sample will be extracted and the concentration of the C-reactive protein in each will be calculated within one assay conducted. From this concentration a standard deviation and mean will be generated. The intra-assay variation will then be calculated as the standard deviation expressed as a percentage of the mean.
Inter-assay variation, for this coefficient a representative sample that has been assayed previously will be chosen and this sample will then be assayed, in duplicate, during five different assays. Again, for these a standard deviation and mean will be generated and the inter-assay variation calculated as the standard deviation expressed as a percentage of the mean.
Test of parallelism, in order to rule out the possibility of other compounds interfering with the assays, in a specific or non-specific fashion, tests of parallelism will be performed on representative samples which have exhibited high and low concentrations. The representative extracts will be diluted with the kit supplied ELISA wash buffer to give a two-fold, four-fold and in some cases where C-reactive protein concentration is measured to be high, an eight-fold dilution. These samples will then be assayed as normal to give concentration values for each dilution. The results from the assay will then be graphed using Excel® and comparison with the standard curves will be made with regression analysis using Minitab statistical analysis package.

These validation tests will be conducted to address the first aim of this project which is to validate the suitability of commercially available ELISA kits for C-reactive proteins for serum and faecal samples from captive pinnipeds, using the Australian Sea Lion (*Neophoca cinerea*) as the initial species for validation purposes. This then allows the second phase of the research to continue. A small group of animals will be used again for some initial trials where they will be closely monitored in different situations including normal and also unfamiliar husbandry situations. For example, before during and after a period of normal routine training of an animal and then during the training of a new unfamiliar routine. The situation where an animal is unfamiliar with what is occurring should cause mild stress within these animals. This mild stress is to mimic the reactions of the body that would be shown in the early disease state. (See Figure 1 above). This is because the situation of where an animal is under stress causes release of glucocorticoids which feed into the liver reaction the same as a disease response. This will further validate the usefulness of these kits for long term monitoring of animals as it will indicate if changes, as has been observed in other animals, occurs in the pinnipeds under these situations. This will then allow these markers to be used as indicators of early disease processes occurring within these animals.

The concentrations of C-reactive proteins for individual animals will be graphed over time to show how concentrations can vary during different events Correlation between concentrations of C-reactive proteins and health or stress situations will be made using non-parametric analysis. As mentioned above regression analysis will be used to test parallelism on the selected samples and all samples will be subjected to analysis of variance to show that the variations observed over the period of the research are actual variations and not just due to "noise" within the data.

Timetable (monthly projection for year of project)

Month 1: Initial contact with institutions involved to confirm project and requirements for the collection procedures and also send sample collection materials to them. Initial testing of a small group of animals from Adelaide and initial testing of samples collected to complete the initial validation process.

Months 2-10: Set up situations for small group testing of animals under different husbandry situations to create periods of stress to mimic early disease situations and complete second aim of project.

Month 10-12: Completion of assays and analysis of data. Begin reports and writing paper for submission to scientific journal

Justification of Budget

As stated above in the research plan in order to give the best correlation between events and C-reactive proteins there is a need to collect as many samples as possible. Therefore the costing of $523 covers all the samples collected over the research period and transport of these to CSU. This is because samples collected are processed at CSU and are not able to be done at the individual institutions therefore this costing must cover all the costs of shipment of these sample to CSU where they can be stored safely for a long period of time.

The assay kits that are available have 96 wells, thus 96 samples can be processed at one time. Within each kit there are six calibrators and when done in duplicate means that 12 wells are used to obtain a calibration curve overall this means that potential because of duplicates each kit will be able to process 42 samples. In order to maximize sample analysis and perform the entire required validation tests there is a need to process as many samples as possible therefore 5 kits will be purchase to be within the budget as each of these kits has been quoted from Laboratory Diagnostics, Kurnell, NSW at $1000 each.

The samples that are assayed also need some minor processing before they are suitable for the assay. This cost includes disposable pipettes and other plastic ware involved in the sample preparation. The cost is estimated at $500 as some of the materials needed are already available from previous work by the principal investigator.

Publications/Dissemination of Results

The initial report will be submitted to CSU once all the data is obtained and analysed. This is envisaged to be within 12 months of the initial start of the project. It is also envisaged that preliminary data will be presented at a Wildlife disease conference that is due to be held in September 2009, with full results being presented at the subsequent year's conference or at another relevant conference should the opportunity arise. At completion of the work findings will also be presented within our school seminar series.

It is envisaged that the collected data and findings will generate 1-2 papers to be submitted to international peer reviewed journals such as the "Journal of Wildlife Diseases" or "Wildlife Research" within 6-12 months of completion of the work

Further presentations over the period will be made to the institutions involved and interested professionals within these so that they can be shown the relevance of this testing to their own and other institutions and to encourage possible future funding and research potentials.

Statement of Outcomes

There are a number of initial and immediate outcomes and some projected ones that would bring benefit to CSU as well as institutions involved within the research.

1. The validation of simple ELISA kits to allow the measurement of C-Reactive proteins in faecal and serum samples of captive pinnipeds will ultimately allow the processing of these samples to be carried out routinely in our laboratory and bring in extra funding for this work to be carried out.

2. It is envisaged that once validated further funding will be applied for and if successful a wider surveillance of captive pinnipeds will be carried out. This will be a coordinated effort to monitor these animals for early disease status and thus alleviate losses due to treatments that may have been prevented by early intervention. This is a cost benefit to the institutions involved and will bring a wider recognition to the school and CSU in this field.

3. National and international funding will then be sought to further fund the use of these markers as a tool to monitor disease and early disease threats in the wild populations of pinnipeds that inhabit our coastlines. This bringing further funding and international recognition to the school and CSU

Track Record

The primary investigator, Geoff Dutton is an early career researcher taking up a lecturing position in Veterinary Anatomy early in 2005. During the past years at CSU there has been a large involvement with the setting up of all facets of the Veterinary Anatomy curriculum for the veterinary students and then the initial presentation of this course to the students. This process has been very time consuming leaving little time for research activities. Furthermore the availability of research funding has not at this stage presented itself although a number of grant applications have been made. Therefore the following material represents achievements prior to commencement at CSU and is presented to substantiate that the principal investigator is able to conduct and report independent research in the area of the proposed project. Furthermore, much of the previous work involved validation of assays within novel species providing the principal knowledge and expertise which is a foundation for the work which is to be conducted with the present funds being applied for.

1997-2003 Ph.D. Thesis, Methods of Assessing Marine Mammal Reproduction, University of Sydney, NSW, Australia **.**Supervisors: Professor MM Bryden and Associate Professor GM Stone

This was mainly an investigation of the reproductive cycle of the Australian sea lion (*Neophoca cinerea*) by the use of faecal steroids. Faecal samples were collected at regular intervals from captive animals and concurrently reproductive events were determined by observations of behaviour of the animals or known reproductive status. A significant correlation between faecal steroid concentrations as measured by radioimmunoassay (RIA) and reproductive events has been demonstrated in a number of species and this observation held true for this species, where concentrations of reproductive hormones correlated significantly with reproductive events. It was also shown that for this species of pinniped non-gravid females might enter a pseudopregnancy cycle that mimics that of the pregnant females within a group.

Samples from the New Zealand sea lion (*Phocartos hookeri*), which included faeces, blood and saliva, were taken from females during the breeding season. Again RIA results indicated that the concentration of reproductive hormones from these samples could be correlated with the reproductive status of the animal. These samples were obtained during an international collaborative research project with Dr. Padraig Duignan and members from IVABS, Massey University, New Zealand

Further collaborative research was conducted with Dr. John Arnould, from the University of Melbourne on concentrations of reproductive hormones in serum of female Australian fur seals (*Arctocephalus pusillus doriferus*). This work was done to further elucidate the reproductive pattern in this species.

A further study using serum, faecal, urine and ice urine (urine voided onto ice) samples collected from Weddell seals (*Leptonychotes weddellii*) from Antarctica by members of the Australian Marine Mammal Research Centre (AMMRC) were used to investigate reproduction within this species. Again hormone concentrations could be measured by RIA and related to the reproductive status of the animal.

In order to complete the marine mammal picture the Bottlenose dolphin (*Tursiops truncatus*) was used as an example of a cetacean. In this instance faeces and blood were collected from captive animals of known reproductive status. The reproductive hormone concentrations from these samples, like those from other species, were found to be highly correlated with the reproductive status of the animals concerned at the time of collection. These types of samples are highly difficult to collect from free-living animals. For this reason, blubber samples from post-mortem species were obtained and used to determine the concentrations of reproductive hormones from these animals. These studies were conducted in an attempt to use blubber hormone concentrations to estimate the blood concentrations of these hormones.

This study complemented the other studies and extended the work completed during the study for the Postgraduate Diploma in Science. Although reproductive hormones could be easily measured, variability of results from the samples proved problematic and more work will be needed to make a definitive conclusion about the validity of this method for assessing an animal's reproductive status.

Grant funding obtained

2002 Massey University Research Fund, $7,480, for "Reproductive anatomy and physiology of the female New Zealand sea lion."

2002 McGeorge Research Fund/Lewis Fitch Veterinary Research Fund, $5000, for "The gross anatomical and histological features of the female reproductive tract in the New Zealand sea lion."

2000 Sea World Research Funds grant, $5,000, for "Pilot study on the concentrations of reproductive hormones in the faeces of the Australian sea lion"

Publications

Refereed Journal Articles

Connolly J.H., Alley M.R., Dutton G.J. and Rogers L.E; (2006). Infectivity and persistence of an outbreak strain of Salmonella enteric serotype Typhimurium DT160 for house sparrows (Passer domesticus) in New Zealand, *New Zealand Veterinary Journal*, **54(6)** 329-332

***Constable S., Parslow A., Dutton G, Rogers T. and Hogg C**., (2006). Urinary Cortisol Sampling: A Non-Invasive Technique For Examining Cortisol Concentrations in the Weddell Seal, Leptonychotes weddellii. *Zoo Biology*, **25**:137–144

Book section

Connolly, J.H. & Dutton, G.J. (2000). "Zoonoses". Proceedings of the Seminar – Wildlife Health in Conservation Publication No. 204, Veterinary Continuing Education, Massey University, Palmerston North New Zealand

Oral Conference Presentations

"Concentrations of progesterone in the serum, milk and faeces of the female New Zealand sea lion (Phocarctos hookeri) from the Auckland Islands". Joint International Conference of World Association of Wildlife Veterinarians, Wildlife Disease Association (Australasia section), Australian Association of Veterinary Conservation Biologists and Wildlife Society of the New Zealand Veterinary Association, Taronga Zoo, Sydney, 1-6th July 2001

"Concentrations of progesterone and oestradiol in faeces of captive female Australian sea lions (Neophoca cinerea)." Southern Hemisphere Marine Mammal Conference, Cowes, Phillip Island, Victoria, Australia, May 2001.

"Concentrations of progesterone in the serum, milk and faeces of the female New Zealand sea lion (Phocarctos hookeri) from the Auckland Islands". Joint conference of the Wildlife Disease Association (Australasia section) and Wildlife Society of the New Zealand Veterinary Association, Marine Field Centre, Goat Island Marine Sanctuary, Leigh, New Zealand, December 2000.

"Preliminary results of a study on faecal steroids of the Australian sea lion (Neophoca cinerea)." Wildlife Disease Association (Australasian section) Annual Conference, Calperum Station, Bookmark Biosphere, Renmark, South Australia, July 1998

Refereed Abstracts/Poster presentation

Dutton, G.J., Bryden, M.M., & Stone, G.M. (1999). Preliminary results of a study on concentrations of progesterone and oestradiol in faeces of the female Australian sea lion (Neophoca cinerea). *Proceedings of the 13th Biennial Conference on the Biology of Marine Mammals, Maui Hawaii. USA*

Papers in preparation

Dutton, G.J., Bryden, M.M., & Stone, G.M. (2008) Assessment of the reproductive status of the female Australian sea lion (*Neophoca cinerea*) by measurement of reproductive steroid concentration in faeces. *Wildlife Research.*

Dutton, G.J., Bryden, M.M., & Stone, G.M. (2008) "Assessment of the reproductive status of the male Australian sea lion (*Neophoca cinerea*) by measurement of reproductive steroid concentration in faeces." *Wildlife Research*.