Archives of Environmental Contamination & Toxicology (AECT)
Received: 6 July 2012 / Accepted: 24 February 2013
DOI 10.1007/s00244-013-9885-5

Running Title
T-cell response in oiled aquatic birds

Title

T-cell responses in oiled Guillemots and Swans in a Rehabilitation Setting.

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Key Words
Swans Guillemots PAHs Immunotoxicity Oil Diesel

Abstract

Aquatic birds are commonly affected by oil spills. Despite rehabilitation efforts the majority of rehabilitated common guillemots (*Uria aalge*) do not survive, while mute swans (*Cygnus olor*) tend to have higher post-release survival. Polyaromatic hydrocarbons (PAHs) present in crude oil and diesel are immuno-toxic in birds affecting cell-mediated responses to immunogens. Being a target of PAH toxicity, T-lymphocyte response to controlled mitogen administration (phytohemaglutinnin – PHA test) was investigated in a scoping study, as a potentially useful minimally-invasive *in vivo* test of cell-mediated immunity. The test was conducted on 69 mute swans and 31 common guillemots, stranded on the Norfolk and Lincolnshire coastline and inland waterways in England (U.K), either due to injury or contamination with crude oil or diesel. T-lymphocyte response was significantly reduced in swans with higher oil scores. T-lymphocyte responses were also reduced in guillemots but the finding was not statistically significant.

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1. Introduction

Stranded aquatic birds are a common feature of oil and diesel spill events. Birds become externally contaminated on contact with oil at the water's surface, ingest significant quantities of oil by preening resulting in oral exposure to polycyclic aromatic hydrocarbons (PAHs) (Troisi and Borjesson, 2005). There are considerable efforts to recover, clean and rehabilitate oiled aquatic birds for release to the wild. In the UK, post-release survival of rehabilitated oiled common guillemots (*Uria aalge*) is very low, while survival of mute swans (*Cygnus olor*) contaminated mainly by localised incidental diesel spills in marinas and inland waterways, tends to be much higher (Wernham et al. 1997). This is because guillemots are more sensitive to captivity and handling stress in rehabilitation than larger more robust species like swans. Also guillemots are usually oiled on contact with expansive areas of spilt crude oil in open sea or coastal areas leading to severe oiling of the plumage and subsequent oral exposure from preening (Grantham, 2004). On the other hand, swans are usually oiled on contact with less toxic and less viscous diesel from smaller localised spills in inland waterways. Beyond the immediate influence of the acute effects of oiling (weight loss, hypothermia), there are long term chronic effects of PAH contamination, such as reproductive toxicity, thyroid disruption, hemolytic anaemia, hepatic and renal toxicity which pose further threats to the long term survivability of aquatic birds which survive initial oiling (Peakall et al. 1981; Troisi et al. 2007; Leighton, 1993).

Post-release survival of rehabilitated oiled birds in the wild is partly dependent on resistance to pathogens which may be compromised by PAH immunotoxicity (Briggs et al. 1996). Controlled dosing of gulls and ducks with crude oil have shown that there is a shift away from granulopoiesis to erythropoiesis to combat PAH-induced hemolytic anemia, leading to reduced resistance to infection and spleenic and bursal atrophy (Holmes et al. 1978; Rocke et al. 1984; Leighton, 1986). Cell-mediated immunity is the main target for PAH immunotoxicity while humoral immunity (antibody production) is less significantly affected (Rocke et al. 1984). Captivity and handling stress also influence immune competence in rehabilitated oiled aquatic birds, potentially exacerbating PAH-associated immunosuppression (Newman et al. 2000). The immediate impact of immune suppression on rehabilitated birds is reduced resistance to infections which spread quickly in indoor environments where large numbers of birds are in close proximity particularly following a large oil spill. For example, *Aspergillosis spp* infections of the lung are frequently reported and infection of injuries such as hock lesions which develop from standing on unsuitable substrates. Furthermore, inflammation of the intestinal mucosa due to oil ingestion, also results in nutrient mal-absorption resulting in weight loss, diarrhoea and reduced intestinal T-lymphocyte production allowing greater pathogen entry across the gut, also influencing survivability (Newman et al. 2000; Briggs et al. 1996).

Biomarkers are useful tools for assessing exposure and effects of pollutants in wildlife. They constitute a biological response(s) that is dose-dependent with toxicant exposure. Biomarkers can be used to monitor pollutant exposure and/or effects and are intrinsically linked to the toxic mechanism by which they cause effects. For a biological response(s) to be a suitable biomarker for monitoring PAH exposure and associated PAH-induced health effects in live oiled wildlife it must be measurable in samples collected non-destructively (e.g. blood). Candidate biomarkers must also be sufficiently specific and sensitive to monitor environmental exposures and induced by a known toxic mechanism. Tests evaluating changes in immune tissue/organ histo-pathology, plasma thyroid hormone, retinol, cell-mediated (T-cell response, differential leucocyte counts) and humoral (immunoglobulin concentrations) responses provide information on how contaminants interfere with immune function and have been used in avian biomonitoring studies (Peakall et al. 1981; Grasman et al. 1996; Newman et al. 2000; Smits et al. 2002; Troisi et al. submitted). The phytohemagglutinin (PHA) skin test is a simple non-destructive *in vivo* method to study how T-lymphocyte response to mitogenic challenge may be altered due to contaminant exposure (Grasman, 2002). Indeed this test has been validated by controlled dosing studies with polychlorinated biphenyls in birds and has been successfully applied in biomonitoring studies of organochlorine exposure in wild bird populations (Grasman et al. 1996; Smits et al. 2002; Sagerup et al. 2009).

Humoral responses have been found in both biomonitoring and dosing studies to be less useful biomarkers of immunotoxicity since the primary target of immunotoxic pollutants (PAHs and PCBs) is cell-mediated immunity. Furthermore, immunoglobulin concentrations are more susceptible to confounding factors (e.g. inflammation, dehydration, nutritional status, age and sex) (Grasman et al. 1996; Briggs et al. 1996). Non-destructive measurement of humoral immune response requires species-specific immunoglobulin assays which are not yet commercially-available for birds. However, the PHA test has potential to be a useful cost-effective, non-destructive tool for assessing cell-mediated (T lymphocyte) immune competence in a rehabilitation setting. To investigate this stranded/injured mute swans and common guillemots admitted to East Winch RSPCA Wildlife Centre, many of which had been exposed to crude or diesel oils, were subjected to PHA testing as part of a scoping study to establish reliability and practicality issues with using the test on smaller and larger species of oiled birds in a rehabilitation setting.

2. Methods

Stranded adult common guillemots (*Uria aalge*) and adult mute swans (*Cygnus olor*) admitted to the East Winch Wildlife Hospital (Royal Society for Prevention of Cruelty to Animals; RSPCA) were PHA tested by veterinary staff within routine treatment under UK Home Office Licence (sampling and treatment of live animals for their veterinary care). The guillemots were stranded along the Norfolk or Lincolnshire coastline as a result of injury or contamination with crude oil from the Tricolour spill in The French Channel (Grantham, 2004), while swans were stranded in marinas and inland waterways in the local area from injuries or diesel contamination. Stranded birds admitted to the hospital for various reasons (e.g. injury, oiling, infection) over a period of a 4 week period between December and January in winter. It was not possible or ethical to obtain birds from the wild specifically for this study. It was not possible to achieve equal or adequate representation for each level of oil exposure, body condition, outcome (euthanised or released), age or sex. The uncontaminated birds in this study were admitted due to physical trauma/injury.

All birds were in various states of stress, dehydration and emaciation. Body weights were recorded on admission. Overall body condition was scored on the basis of the availability of fat reserves on an arbitrary basis by eye, as follows; Emaciated (0); lean (1); fair (2); good (3). External oiling of plumage was also scored on an arbitrary scale by eye, as follows; unoiled (0), lightly oiled (1; <50% coverage), moderately oiled (2; 50-75% coverage), heavily oiled (3; < 75% coverage). Information on sex of the swans was not collected by the RSPCA and so was not available to this study. Some of the birds that died were subject to *post mortem* examination to determine sex and age class (guillemots only) and to investigate the cause of death. The majority of the guillemots died by euthanasia due to the severe effects (including haemolytic anemia, muscle wasting, renal dysfunction) of worsening emaciation and cachexia (n=15). A low number died or were euthanised due to physical injury (n=2), or conditions such as hock lesions (n=2) and *Aspergillosis spp* infections (n=4) contracted well after admission as a result of being held in captivity which is a commonly observed phenomenon in oiled bird rehabilitation centres (Balseiro et al. 2005).

To test T-lymphocyte response, the PHA test was conducted on live guillemots and swans once they had been washed and stabilised following admission (1-3 days from admission; any birds with infection were excluded from the study). This involved the injection of mitogen (100µg of PHA, dissolved in sterile 0.1ml Hartmann's solution) into the right foot web to generate an immune response and 0.1ml sterile Hartmann's solution into left foot web as a control. Quadruplicate measurements of foot web thickness at the injection site were undertaken with digital callipers (accuracy ±0.01mm) immediately prior to injection and +24 hrs. The mean change in foot web thickness (in mm) from the time of injection and after 24 hours was calculated as the "PHA Response" subtracting any change in foot web thickness observed in respective controls. Normally the patagium (wing web) is used for PHA injection in birds in an area from which feathers have been removed (Grasman, 2004). However, most of the birds in this study were in a distressed condition, so veterinary staff decided it more appropriate to inject the foot web (inter-digital skin) which is more accessible, thereby minimising handling and further distress. This approach has been used successfully to assess immune function of juvenile chickens exposed to polychlorinated biphenyl due to their small body size (Lavoie et al. 2007). It was not possible to test healthy unoiled guillemots in this study due to the restrictions within the UK Home Office licence under which the RSPCA staff were operating, which prohibits wild animals being sampled unless they require veterinary care.

3. Results

PHA cell responses were reduced 3-fold in the most contaminated swans (score 3) compared with unoiled swans (figure 1). Paired comparison of mean data for each oil score category, revealed statistically significant reductions in response according to two-tailed student *t*-test as follows; 0 v 1 ($p<0.05$); 0 v 2 ($p<0.01$); 0 v 3 ($p< 0.001$); 1 v 3 ($p<0.01$); 2 v 3 ($p<0.01$), with the exception of scores 1 v 2. In guillemots, PHA response was depressed in heavily oiled birds (score 3) compared with unoiled birds (score 0), but differences between category means were not statistically significant (figure 1). Body weight and PHA response in swans and guillemots according to sex, age class, body condition and outcome are presented in Table 1. Although not statistically significant, PHA response was less pronounced in females compared with males (age classes pooled). PHA responses were similar between age classes for both species (sexes pooled). There were no significant differences in PHA response in guillemots on the basis of outcome and since all swans survived, comparison of PHA response between outcomes could not be made. Swans with body condition score 2 had significantly greater body weights than birds with score 1 ($p<0.001$) - no swans represented categories 0 and 3. Although not statistically significant, body weights of guillemots with body condition score 1 were also heavier than those with poorer body condition scores - no guillemots represented categories 2 and 3. In figure 2, body weight and PHA response from both species are shown to be positively associated, but the correlations were not statistically significant.

4. Discussion

The PHA test was found to be a reliable method for measuring T-cell mediated responses in the foot web of oiled swans. The significantly lower level of swelling response measured in the foot wed of heavily oiled swans was indicative of an immunosuppressed response. A reasonable explanation for this is the immunotoxicity exerted by the PAHs to which the birds were exposed. That the PHA foot web test was also successful for assessment immune-competence of guillemots in this study but there was a lack of a statistically-significant association between oil exposure and PHA response mainly due to insufficient representation of each oil score category causing confounding factors to have great influence. The results of the PHA test were also compromised to some degree by the extreme level of emaciation and dehydration in the guillemots leading to poor circulation and cold legs and feet. Poor vascular circulation may have reduced T-cell delivery to the target site of PHA injection thereby influencing changes in foot web thickness. This problem this posed for testing was overcome by warming the bird's legs to boost poor circulation and soften the skin which enabled reproducible injection and measurement. However, the exacerbated condition of the guillemots would no doubt have suppressed the PHA response. This is also the reasoning behind selecting the foot web skin as the PHA injection site instead of the wing web, as it is better suited for smaller birds like chicks and smaller species which are more easily stressed by handling (Lavoie et al., 2007). Most of the guillemots in this study did not respond to treatment and died (28 of 31 birds) within a few months of admission due to their level of emaciation. Unfortunately, "normal T-lymphocyte" responses to PHA from healthy guillemots are not available in the published literature to provide control data and it was not possible under the UK Home Office Licence under which the RSPCA were operating to capture healthy guillemots or swans to participate as controls for this study.

The only comparable study of avian cell-mediated responses following exposure to petroleum chemicals is the controlled dosing of zebra finch chicks (*Taeniopygia guttata*) to consolidated oil sands tailing (CT) water, where the lack of any immunosuppressive effects was proposed to be due to the low PAH concentrations found in CT water (< 2µg/l; Madill et al., 2001) and short duration of exposure (Smits & Williams, 1999). This in contrast with what the immunosuppressive effects observed in this study. An explanation for this, are the different exposure scenarios. Birds in this study were exposed to comparatively higher concentrations of PAH by preening crude or diesel oil from their feathers, over a period of days or weeks, where petroleum oils contain significantly greater quantities of PAHs than tailing water (Madill et al. 2001). Suppression of PHA responses associated with organic contaminant exposure observed in this study, was in agreement with PCB-induced suppression observed in Great Lake Caspian Terns (*Sterna caspia*) and Herring Gulls (*Larus argentatus*), where 30-45% reduction in responses was observed in heavily contaminated populations (Grasman et al. 1996). Furthermore, in controlled PCB dosing studies with American Kestrels (*Falco sparverius*), PHA responses were also found to be significantly reduced (Smits et al. 2002).

T-cell response in both swans and guillemots was generally greater in individuals with higher body condition scores but differences between scores were not significant owing to insufficient representation of each category. It has already been established that body mass is positively correlated with T-cell response in unexposed birds (Alonso-Alvarez & Tella, 2001). The lack of any relationship between body mass or body condition score and PHA responses in swans may strengthen the possibility that oil exposure was the predominating factor influencing T-cell response in this study. However, since starvation reduces peripheral T lymphocyte activity and inflammatory response, it is plausible that the emaciated condition apparent in most of the guillemots suppressed their T-cell responses. Indeed the immunosuppressive effect of emaciation, will likely influence recovery of free-living oiled seabirds in the wild, since emaciation this is the predominated immediate impact of oiling (Wanless et al., 2000). In the case of age or sex-dependent differences in PHA responses, no statistically-significant differences were observed in guillemots due to small sample size and lack of complete age and sex data in this study. It was not possible to examine the influence of age and sex on PHA response in swans as age and sex data were not recorded. It is important to undertake further studies with larger sample sizes to investigate whether juvenile and adult female birds, particularly juvenile females, are more sensitive to the immunosuppressive effects of PAHs than other members of the population due to their importance to breeding and population recovery. In terms of differences in

PHA response with outcome (released/died), no conclusions could be made because of the lack of representation from each outcome category (majority of the guillemots died and all the swans were released). The greater survivorship of swans compared with guillemots can be explained as follows. Firstly, swans were exposed to diesel not crude oil as were the guillemots, which is less toxic (contains a lower proportion of PAHs). Also being slightly more water soluble, diesel is easier to successfully wash off the plumage. Due to the difference in exposure and the fact that swans are a larger more robust species, oiled swans respond much better than guillemots to rehabilitation.

Although the PHA test provided an indication of the responsive capacity of cell-mediated immunity targeted towards a specific mitogen, the PHA test is not able to assess the integrity of the functionality of these cells. Despite this, the PHA test was shown in this study to be useful for cost-effective and minimally-invasive means of assessment of cell-mediated immunity in a rehabilitation setting. One of the roles of well-differentiated T-cells is to seek and destroy antigens and this study provides some evidence that oil exposure may reduce T-lymphocyte immune response. However, further validation is necessary with the testing of healthy control birds to qualify the dose-response relationship of PHA response to confirm its suitability as a biomarker of oil-induced PAH immunotoxicity. In the rehabilitation context, handling and captivity stress may have influence on PHA response albeit minor compared with the influence of infections such as *Aspergillosis spp* and hock infections which can occur in captivity (Balseiro et al., 2005). Therefore, PHA testing is best applied to stabilised rehabilitated guillemots that have re-gained their weight, as a means to establish if individuals are adequately immune-competent prior to release to improve their chances of survival. Also by this time, rehabilitated birds would have acclimatised to handling and captivity so the influence of handling stress on the PHA test results should be minimal.

4. Conclusion

PAHs affect cell-mediated immunity to a greater degree than humoral immunity (Rocke et al. 1984) and the PHA test has been successfully used to assess immunotoxicity of pollutants on T-lymphocyte responses in various bird species (Grasman, 2002). The PHA test is cost-effective, simple to use and minimally-invasive and this scoping study showed that by boosting leg blood circulation and using the foot web as the PHA injection site, the PHA test can provide reliable immune competence assessment of even smaller, stressed and emaciated oiled seabirds suiting the requirements of wildlife rehabilitation centres. Although only a preliminary investigation, the PHA test was used in this scoping study to measure T-lymphocyte response in stranded swans and guillemots exposed to varying degrees with diesel and crude oils in a wildlife rehabilitation centre. The study found that immune-competence of swans was significantly reduced in birds with higher oil scores but no such conclusion could be drawn with guillemots due to the influence of various confounding factors (emaciation, dehydration, stress) and inadequate numbers of control (unexposed) birds in this study. The impacts of severe emaciation and dehydration from oiling on this sensitive species make it more difficult to measure small changes in tissue inflammation as a biological response. Consequently, the PHA test is best suited to test guillemots with good body condition, for example to confirm T-lymphocyte integrity prior to release in rehabilitated birds. The overall influence of oil-induced immunosuppression on survivability of rehabilitated oiled birds is not known. At the population level, aquatic birds face numerous ecological and toxicological challenges ranging from oil spill disasters, food chain contamination (organic & heavy metal pollutants), overfishing, pathogenic challenges and climate change, to mention a few. A subtle change in cell-mediated immune competence due to PAH exposure, may exacerbate these impacts and is worthy of further study to improve rehabilitation practice and survivability.

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	Mean +/- std. error (range)	
	Body Weight **(kg)**	***T-Cell Response*** **(mm)**
Mute Swans (n = 69)	6.79 +/-0.46 (3.60 - 10.2)	0.44 +/-0.07 (-0.23 – 1.15)
Sex	n/d	n/d
Age Class		
Adults	8.07 +/-0.60 (5.70-12.10) (n=15)	0.39 +/-0.06 (-0.23-1.11) (n=34)
Juveniles	6.13 +/-0.82 (3.60-7.90) (n=6)	0.42 +/-0.08 (0.01-1.10) (n=14)
Body Condition		
Score 0	(n=0)	(n=0)
Score 1	5.98 +/- 0.31 (3.60-6.90) (n=13)	0.37 +/-0.06 (-0.10-1.11) (n=31)
Score 2	10.00 +/- 1.21 (7.90-12.10) (n=4)	0.32 +/-0.12 (-0.23-0.94) (n=12)
Score 3	(n=0)	(n=0)
Outcome	n/a	n/a
Common Guillemots (n = 31)	0.73 +/-0.04 (0.60 - 0.86)	0.02 +/-0.01 (-0.10 - 0.16)
Sex		
Males (n=20)	0.74 +/-0.02 (0.61 - 0.86)	0.03 +/-0.01 (-0.10 - 0.16)
Females (n=11)	0.71 +/-0.03 (0.60 – 0.83)	0.01 +/-0.02 (-0.09 - 0.11)
Age Class		
Adults (n=21)	0.77 +/-0.02 (0.61 - 0.86)	0.02 +/-0.01 (-0.10 - 0.13)
Juveniles (n=10)	0.64 +/-0.01 (0.60 - 0.71)	0.02 +/-0.02 (-0.01 - 0.16)
Body Condition		
Score 0 (n=3)	0.66 +/- 0.28 (0.31-0.71)	-0.02 +/-0.02 (-0.05-0.02)
Score 1 (n=28)	0.74 +/- 0.16 (0.60-0.86)	-0.02 +/- 0.01 (-0.16-0.10)
Score 2 (n=0)	-	-
Score 3 (n=0)	-	-
Outcome		
Died (n=28)	0.72 +/-0.18 (0.60-0.86)	0.03 +/-0.01 (-0.10-0.16)
Released (n=3)	0.77 +/- 0.28 (0.72-0.81)	0.05 +/-0.04 (-0.02-0.11)

Table 1. Species, age class and outcome related means (+/- std. error) for body weight and T-cell response (change in foot-web thickness). Abbreviations: n/a = not applicable (as all were released); n/d = not data available.

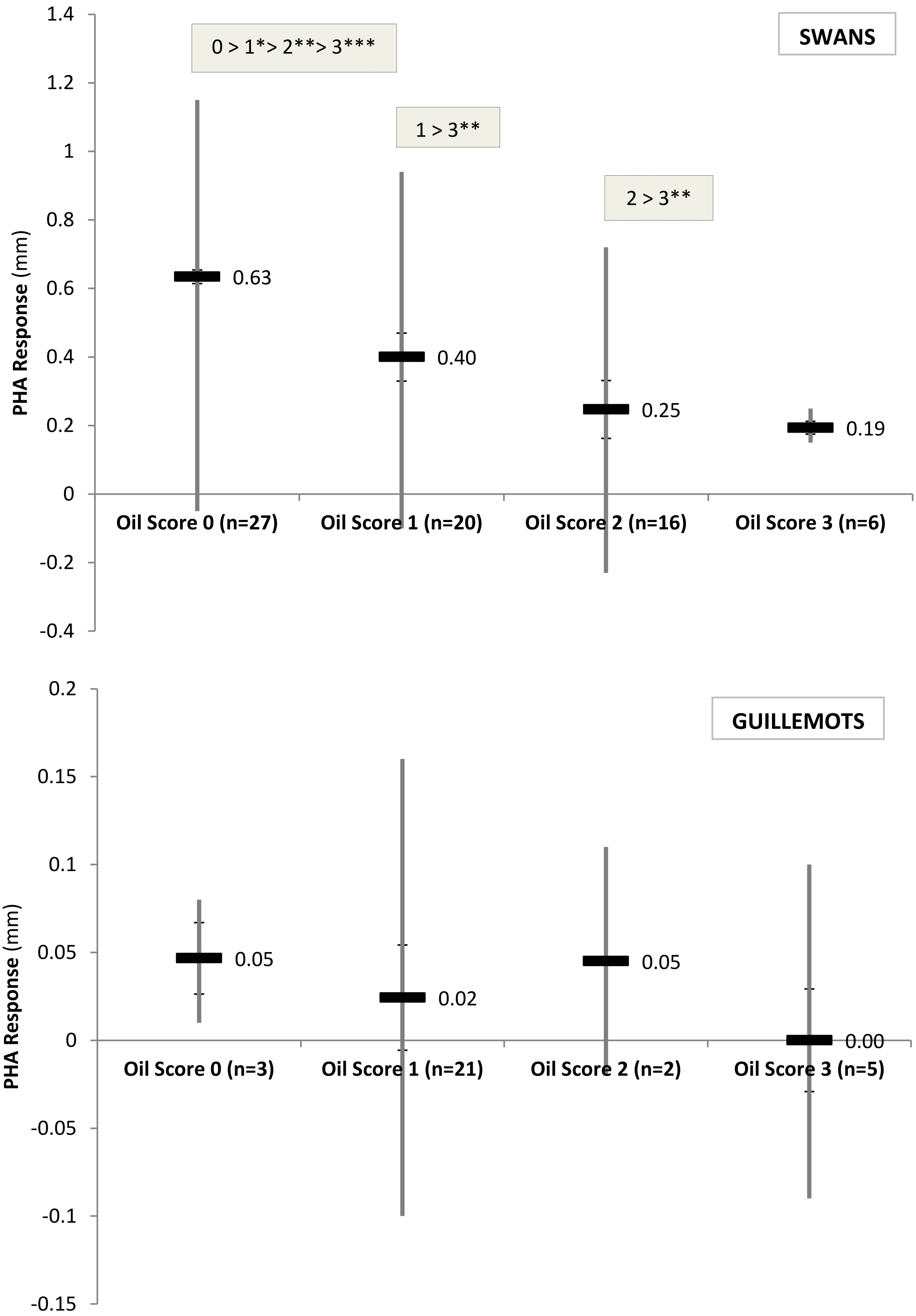

Figure 1. Relationship between exposure and PHA response in mute swans (A) and common guillemots (B). The PHA response was calculated as change in mean foot web thickness minus change in control foot expressed here as means (± standard error) and range values. Where present, statistically significant differences between categories are indicated (* p<0.05; **p<0.01; ***p<0.001).

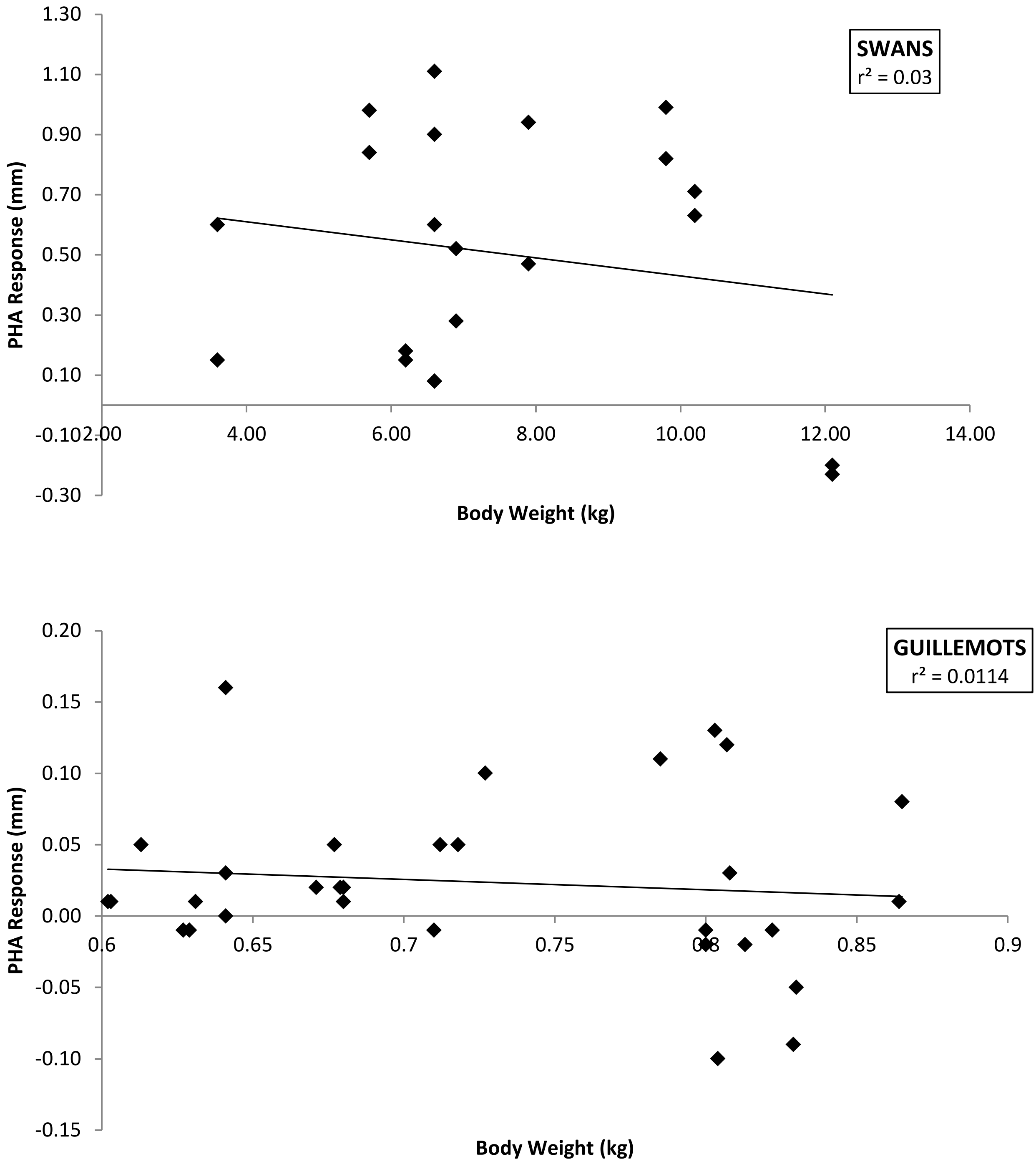

Figure 2. Relationship between body weight and PHA response in mute swans and common guillemots. (n.s. = not significant).