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This is the final peer-reviewed accepted manuscript of: S. M. Hoseini, S. Namroodi, A. Zaccaroni, A. Sayad-Shirazi, M. Pérez-López, and F. Soler-Rodríguez: Detection of Carcinogenic Polycyclic Aromatic Hydrocarbons in Stranded Caspian Seals (*Pusa caspica*). AQUATIC MAMMALS Volume 46 (1) Pages 58-66. The final published version is available online at: https://doi.org/10.1578/AM.46.1.2020.58

Detection of Carcinogenic Polycyclic Aromatic Hydrocarbons in Stranded Caspian Seals (*Pusa caspica*)

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Abstract

Polycyclic aromatic hydrocarbons (PAHs), which contain many carcinogenic compounds, are a major ingredient of petroleum/oil. PAH pollution of the Caspian Sea, the world's largest lake, is rapidly occurring and may be affecting the endangered Caspian seal (*Pusa caspica*), the only marine mammal in this lake. To analyze the entrance of PAHs into the Caspian Sea food chain and the health status of Caspian seals, we measured 16 carcinogenic PAHs in the blubber, kidney, and liver tissues of 10 Caspian seal carcasses from the coastal region of northeastern Iran, using gas chromatography-mass spectrophotometry. Of the 16 PAHs investigated, anthracene, phenanthrene, and naphthalene only were identified in nine sampled Caspian seals. Concentrations of anthracene ($\bar{x}$=84.83±79.86 ppb wet weight [w.w.]), phenanthrene ($\bar{x}$=31.75±52.22 ppb w.w), and naphthalene ($\bar{x}$=25.1±31.57 ppb w.w.) in blubber tissues were higher than in kidney and liver tissues. The concentration of PAHs in tissues was significantly higher in male than in female seals

and we found an inverse relationship between seal age and PAH concentration in tissues. Although no data exist concerning toxic effect concentrations of PAHs in Caspian seals, PAH detection in seal carcasses highlights a potentially stressful condition that may impact the health of Caspian seals and other sea life in this lake. Appropriate strategies for the control of PAH entrance into the Caspian Sea should be sought and studies for the determination of pathogenic and lethal doses of PAHs in Caspian seals should be pursued.

Key words: PAHs, Iran, tissues, *Pusa caspica*

Introduction

The Caspian Sea, the largest closed lake in the world, is surrounded by five countries including Russia, Ghazakhstan, Azerbaijan, Turkmenistan and Iran (Shinsuke et al., 2003). This multinational access, in addition to the high density of passenger and commercial vessels and the influx of contaminated water from the numerous rivers that flow through industrial and agricultural areas, have resulted in the Caspian Sea becoming increasingly polluted (Effimoff, 2000; Kaplin, 1995). Extraction of oil from the lake, especially from the central and northern parts, and the entry of petroleum-based fuels in contaminated water have also contributed to pollution of the lake (Mille, 2007; Tolosa, 2004).

Polycyclic aromatic hydrocarbons (PAHs) with benzene rings are a highly toxic component of oil. Sixteen compounds of PAHs from oil have been identified as highly toxic and mutagenic for animals (Freeman et al., 1990). Generally, PAHs enter the environment in two ways: (a) human activities (pyrolytic origin) such as extraction, refining, transfer and export of crude oil, anthropogenic oil spills and other products, and (b) natural entrance (petrogenic origin) of biological resources through PAH synthesis by plankton, bacteria, algae, and the decomposition

of plants (Nasrollahzadeh Saravi et al., 2013). Fortunately, PAHs break down quickly when exposed to light and oxygen (e.g., photo-oxidation) and some PAHs are biodegraded by naturally-occurring bacteria (Kanaly & Harayama, 2000; Mrozik et al., 2003). PAHs divided in low and high molecular weight based on number of their benzene rings. Oil spills represent one of major threats for marine mammals, both in the short and long term. However, high molecular weight (HMW) PAHs have higher tendency to accumulate in animals' tissues than low molecular weight (LMW) PAHs. In aquatic ecosystem HMW PAHs precipitate faster than LMW PAHs and have lower chance to accumulate in body of marine animals (Lawal, 2017). Limited studies have shown PAH pollution in Caspian Sea coastal areas of Baku (due to oil extraction), northern Russia and Kazakhstan, and southern Iran (Kardovani, 1995; Tolosa, 2004).

The Caspian seal (*Pusa caspica*), the only species of marine mammal in the Caspian Sea, is at the top of the food chain in this ecosystem. This species has migratory life style and due to various factors such as death in fishing nets and reduced reproductive rates in male and female seals because of exposure to pollutants, the health status of this species is poor (Harkonen et al. 2008, ;Watanabe et al., 2002). Mass mortalities of these seals along the Caspian Sea coast during 1997-2000, along with the factors cited above, have resulted in placement of the Caspian seal on the IUCN red list (Goodman & Dmitrieva, 2016). Considering the increase in Caspian Sea oil pollution in recent years and the migratory lifestyle of Caspian seals, we monitored PAH pollution in the Caspian Sea and their health status by measuring and assessing concentrations of 16 mutagenic PAHs in stranded Caspian seal tissues.

Methods

Sampling

After approval of the study by the Ethics Committee of the Deputy of Natural Environment of Golestan Province (permit number: 125/7894), 10 stranded Caspian seals along the coasts of Golestan and Mazandaran provinces, Iran, were collected during 2012-2015. Stranded Caspian seals were trapped in fishing nets and were found fresh by fisheries. Following biometric analysis and clinical examination, the seals were transferred to the laboratory and necropsied. Liver, kidney, and blubber tissues were sampled to measure PAH concentrations and a lower canine tooth was extracted to determine seal age (Amano et al., 2000).

Measurement of PAH concentration

This analysis was performed at the Toxicology Lab, Faculty of Veterinary Medicine, University of Extremadura, Caceres, Spain. To measure tissue concentrations of PAHs, the standard method of Lucas and Zhao (2015) using gas chromatography-mass spectrometry (GC-MS/MS) was applied. The samples were shredded with glass slurry, freeze dried for 72 h, and homogenized using a mortar and pestle. A portion of each freeze-dried sample was weighed into 50 mL centrifuge tubes and MilliQ water was added to obtain the equivalent to 5 g of fresh sample. Ten ml of acetonitrile were added to the tube and the sample was shaken vigorously by hand for 2 min and centrifuged for 5 min at 5,000 rpm. The supernatant was transferred to a 15-ml centrifuge tube containing 1 g of Agilent Enhanced Matrix Removal-Lipid sorbent (Agilent Technologies, Santa Clara, California, USA) and placed on a vortex mixer for 60 s. The solution was then centrifuged at 5,000 rpm for 3 min. The supernatant was transferred to a second 15-ml tube containing 2 g salt (1:4 NaCl: MgSO 4) and immediately vortexed and centrifuged for 3 min. The top layer containing acetonitrile was evaporated to dryness in a rotatory concentrator, reconstituted in ethyl acetate:n-hexane (1:4) and filtered (0.45 micron) into a 2-ml GC vial.

Obtained extracts (1 µl) were injected into a gas chromatograph-mass spectrometer (SCION GC-MS-Triple Quad MS/MS; Bruker Scientific Instruments, Billerica Massachusetts, USA) and PAHs were separated on a 30-m Agilent J&W DB-5ms (5% diphenyl/dimethylpolysiloxane) capillary column (250 µm id. x 0.25 µm film thickness). For quality control and for quantitative purposes, one sample from each matrix (blubber, liver, and kidney) was pre-spiked at 40 ng/g with a certificate standard (DE-PROM 16 EPA Priority PAHs in Toluene from LGC Standards, 100 µg/ml) containing the 16 PAHs considered to be of primary concern by the United States Environmental Protection Agency (USEPA). Qualification and quantification ions were monitored for each compound at m/z = 102+126+127 for naphthalene, 126+150+151 for acenaphthylene, 127+151+152 for acenaphthene, 139+163+164 for fluorene, 152+176+177 for phenanthrene, anthracene, 200 for fluoranthene, 151+200 for pyrene, 202+226+227 for benzo[a]anthracene, chrysene, 250 for benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, 274 for benzo[g,h,i]-perylene, indeno[1,2,3-cd]pyrene, and 276+277 for dibenzo[a,h]anthracene.

Results of each spiked matrix were used for quantitation of matrix-related samples. These matrix-matched samples were chosen in order to compensate for any signal suppression/enhancement compared to their relative response in pure solvent. Procedural blanks containing reagents only were screened during the analysis of each batch (one per tissue) in order to ensure that solvents and all the used material were free from PAHs residues. To evaluate the linearity of the method calibration curves were built with concentrations ranging from 4 to 400 µg/L with ar 2>0.98. The limit of quantitation (LOQ) was determined as the lowest concentration of the compounds which can be reliably detected with the signal-to-noise (S/N) ratio higher than

10. In our study, the LOQ for individual PAHs in samples ranged from 0.5 to 5 ng/g, on a wet weight [w.w.] basis.

Data analysis

Normality and lack of data were evaluated using Kolmogorov-Smirnov test. The result of this test was higher than 0.05, indicating that the data was normal.The effect of sex on the PAH concentration in sampled tissues was investigated using Student's t-tests. Liner regression test was used to survey impact of age on PAH concentration. Also ANOVA one way test was used to survey difference of PAH concentrations in sampled tissues.

Results

Of the 16 PAHs studied, three (i.e. anthracene (Log $K_{OW=}$4.54, octanol-air partition coefficient (K_{AO}) $_{=}$7.55, number of benzene rings (NB) = 3), phenanthrene (Log $K_{OW=}$4.57, $K_{AO=}$7.57, NB=3) and naphthalene (Log $K_{OW=}$3.37, $K_{AO=}$5.19, NB=2) were identified in the tissues of sampled Caspian seals (Table 1) (National Center for Biotechnology Information, 2019). We detected PAHs in nine of the 10 sampled seals at different concentrations and the accumulation pattern of PAHs differed among tissue types. Among blubber tissues, 70% were contaminated with anthracene, 50% with naphthalene, and 40% with phenanthrene. For liver tissues, 80% of samples also were contaminated with anthracene, 80% contained phenanthrene, and 50% contained naphthalene. For kidney tissues 90% were contaminated with anthracene and 40% with naphthalene. Phenanthrene contamination was not detected in kidney tissues (Table 1). The concentrations of anthracene ($\bar{x}$=84.83±79.86 ppb w.w.), phenanthrene ($\bar{x}$=31.75±52.22 ppb w.w.), and naphthalene ($\bar{x}$=25.1±31.57 ppb w.w.) in blubber tissues were significantly (p=0.001) higher than in kidney and liver (Table 2). The concentration of anthracene in all sampled tissues

was significantly (p=0.001) higher than the concentrations of phenanthrene and naphthalene (Table 2). Concentrations of PAHs in liver, kidney, and blubber were significantly higher in male than in female seals (Table 3 & Figure 1). Liner regression test indicated a negative relationship between Caspian seal age (using body length as a surrogate for age) and the concentration of PAHs in the tissues (Figure 2).

Place table 1, 2, and 3 here, Place figures 1&2 here

Discussion

Detection of PAHs in sampled Caspian seals indicated that PAHs entered the Caspian Sea food chain. Also, previous studies have indicated pollution with just anthracene, phenanthrene, Dibenz (a,h) anthracene, fluoranthene, fluorene, and naphthalene in the Caspian Sea (Eskandarpour et al., 2014; Nasrallahzadeh Saravi et al., 2012). Absence of other 13 surveyed PAHs in Caspian seals' tissues can be due to elimination of them in Caspian seals by metabolization or absence of them in Caspian seals' habitat (Meador et al., 1995b). Nasrallahzadeh Saravi et al. (2012) studied the sediment and muscle tissue of fish in the southern Caspian Sea near Astara, White River, Tonekabon, and Amir Abad, Iran. They measured an anthracene concentration of 7.6±4 ppb w.w and dibenzene concentration of 66.6±75 ppb w.w in sediment. Benzo fluoranthene, and benzo pyrene were detected in muscle tissues of several Caspian Sea fish species, including the Caspian kutum (*Rutilus frisii kutum*) with concentrations of 530 ppb w.w. and 96.6 ppb w.w. and the leaping mullet (*Chelon saliens*) at 80 ppb w.w. and 176 ppb w.w., respectively. In another study on fish flour (fish meal) of Caspian Sea sprat (*Clupeonella cultriventris caspia*), naphthalene, fluorine, and anthracene concentrations were measured at 24.66±15.52 ppb w.w., 1.32±1.54 ppb w.w., and 1.1±1.92 ppb w.w., respectively (Eskandarpour et al., 2014). Absence of the other

177 studied PAHs in sampled Caspian seals can be due to short time presence of Caspian seal in sea
178 shore of Iran (Harkonen et al. 2008). As surveyed tissues in previous mentioned studies on fish are
179 not similar with tested tissues in our study, we cannot compare obtained results. However,
180 Compared to the PAH concentration in some of these fish species, the higher concentrations in
181 sampled Caspian seals may be due to higher concentrations of fat in Caspian seal tissue and their
182 greater longevity, allowing for longer bioaccumulation.

183 Unfortunately, there are few similar studies on carcinogenic PAH contamination in marine
184 mammals. Marsili et al. (2001) measured total PAHs in blubber tissues of fin whales
185 (*Balaenoptera physalus*) and striped dolphins (*Stenella coeruleoalba*) and found high levels of
186 contamination in these Mediterranean cetaceans. Marsili et al. (1997) surveyed PAH concentration
187 in liver tissues of South American sea lions (*Otaria flavescens*) in the Plata Sea, Argentina. They
188 detected naphthalene ($\bar{x}$=194±54.07 ppb w.w), anthracene ($\bar{x}$= 0.30±2.270 ppb w.w.), and
189 phenanthrene ($\bar{x}$=27.1±21.75 ppb w.w.) in sampled tissues. Many factors such as level and time
190 of animal PAHs exposure, sex and age of sampled animals, species ability to metabolize PAHs
191 and difference in volume of PAHs entrance in animals' habitat can result in detection of higher
192 concentrations of naphthalene and phenanthrene and lower concentration of anthracene in sampled
193 sea lions than sampled Caspian seals (Meador et al., 1995b). Also, Hellou et al. (1991) examined
194 PAH concentration in muscle tissue of harp seals (*Phoca groenlandica*) in the northwestern
195 Atlantic Ocean. Those specimens were contaminated with phenanthrene, anthracene, and fluorene,
196 and PAH concentration was measured between 10 and 31 ppb lipid w.w. As we did not examine
197 muscle tissues in sampled Caspian seals, we cannot compare our results with results of Hellou et
198 al. (1991) on harp seals.

Of the three PAHs detected (naphthalene, phenanthrene, and anthracene), anthracene occurred at the highest concentrations in sampled tissues while phenanthrene had the lowest concentrations. A different trend in PAH concentrations has been documented in fish species of the Caspian Sea; for example, Eskandarpour et al. (2014) reported higher concentrations of naphthalene than anthracene in Caspian Sea sprat (*Clupeonella cultriventris caspia*). Similarly, Kannan & Perrotta (2008) detected naphthalene in kidney tissues in higher concentrations compared to other PAHs in 81 adult female sea otters (*Enhydra lutris*) from the California coast. Many factors, such as differences in the source of PAHs in sampling areas, chemical condition of the sampling area, number of PAH benzene rings, and tendency for bioaccumulation of PAHs in different species could lead to these differences in findings (Meador et al., 1995b).

As mentioned, tissue accumulation patterns of detected PAHs differed among Caspian seal tissue types. The number of PAH benzene rings is an important variable associated with bioaccumulation of PAHs in Caspian seal tissues. With increases in the number of rings, the hydrophobic and lipophilic properties of PAHs increase. When PAHs enter Caspian seal bodies, they can accumulate in several tissues, but especially in blubber. Microbial degradation of PAHs decreases with increases in the number of PAH benzene rings, so PAHs with more benzene rings can accumulate to higher concentrations in tissues (Landrum, 1989; Landrum & Robbins, 1990; Meador et al., 1995a; Meador, 2003). It follows that higher tissue concentration of anthracene than naphthalene could be explained by the higher number benzene rings of anthracene. Higher anthracene tissue concentration than phenanthrene, which has the same number of benzene rings, might be explained by a higher rate of entry of anthracene into the Caspian Sea.

The higher PAH concentrations in blubber tissues may be explained by the hydrophobic and lipophilic characteristics of PAHs. Eskandarpour et al. (2014) found that bioaccumulation of

PAHs in Caspian Sea sprat was directly related to the concentration of fat in sampled tissues, with higher concentrations detected in tissues with higher levels of fats (Eskandarpour et al., 2014). In contrast, there was no correlation between tissue PAH concentrations and the amount of fat in tissues of Harp seals (Hellou et al., 1991).

We found the lowest PAH concentrations in liver tissues. Most of the PAHs are absorbed through the digestive system and transferred to the liver for detoxification by enzymes, including the P450 family and microsomal enzymes (Engelhardt, 1982; Addison & Brodie, 1984; Marsili et al., 1997; Lee et al., 2005). This detoxification process could lead to lower concentrations of PAHs in liver tissues than blubber or kidney.

A negative correlation between PAH concentration in tissues with the age of sampled Caspian seals was found, indicating the influence of age as a confounding factor on PAH concentrations in our seals. Similarly, studies of Northern pike (*Esox lucius*) and Caspian Sea sprat revealed that animal age can affect PAH bioaccumulation rate with similar trends (Ghorbani et al., 2010; Eskandarpour et al., 2014). As age increases, PAH concentration in tissues can decrease due to an increase in PAH metabolism. Hellou et al. (1991) showed that PAH concentration was higher in younger Harp seals than older seals. They also found that the activity of the metabolizing enzymes, including P450 family enzymes, on PAHs increases with age and can lead to a decrease in PAH concentrations in tissues by age (Hellou et al., 1991). However, Harris et al., (2011) who surveyed Hydrocarbon concentrations and patterns in blood samples of 29 live-captured sea otters (*Enhydra lutris*) from British Columbia, Canada, reported similar hydrocarbon concentrations among different age classes (Harris et al., 2011).

The higher average age sampled females (18.2 y old) than males (14.5 y old), may be one explanation for our finding showing lower PAH concentrations in tissues of female Caspian seals, highlighting the putative biotransformation of PAHs by P450 enzymes. This result supports findings of Addison et al. (1973) and Muyer et al. (1988) in harp seals and ringed seals (*Phoca hispida*), respectively. Those authors suggested that this difference could be due to higher activity of P450 family enzymes in females resulting in a lower PAH concentration in female tissues. Eskandarpour et al. (2014) explained that such results can occur due to maternal transfer of PAHs in fish through egg laying. However, studies by Hellou et al. (1991) and Marsili et al. (2001) on harp seals and cetaceans (fin whales and striped dolphins), respectively, showed no relation between sex and PAH tissue concentrations.

Elimination of organochlorine pesticides in female Caspian seals through giving birth has been described, but there is no information about offloading of PAHs in seals via that mechanism (i.e. maternal transfer) (Tanabe et al., 1978; Subramanian et al., 1987; Nakata et al., 1995). Thus, additional studies are needed on this topic.

Molecular ratios of PAHs have been used to identify their sources in the environment. According to Hajizadeh et al. (2010), if the ratio of phenanthrene to anthracene is >10, the origin of these pollutants is considered petrogenic, and but if the ratio is <10, their origin is considered pyrolytic. Nasrollahzadeh Saravi et al. (2012), who detected PAHs in leaping mullet and Caspian kutum in the southern part of the Caspian Sea (i.e. Mazandaran and Golestan), reported that the origin of PAHs in that region of the Caspian Sea is more pyrolytic. Nemati Varnosfaderany et al. (2014) and Baniemam et al. (2017) reported both pyrolitic and petrogenic origins of PAHs in the south Caspian Sea (Iranian coastal regions). In the present study, the ratio of PAHs with low molecular weight (phenanthrene) to those with a high molecular weight (anthracene) also indicated

that the PAH compounds in sampled Caspian seals may have pyrolytic sources. Nevertheless, Kannan & Perrotta (2008) concluded that the detection of PAHs with a predominance of di- and tri-cyclic PAHs over tetra- and penta-cyclic PAHs (as we also detected in Caspian seals) suggest petrogenic sources.

In conclusion, it appears that Caspian seals are exposed to PAHs of both petrogenic and pyrolytic origins in the Caspian Sea. Our results show that PAHs eventually accumulate in the lake's food chain and enter Caspian seal tissues. Because PAHs retention rate time are low in the environment (i.e. high elimination rate), their accumulation in Caspian seals indicates that the emission or inflow rate of PAH entering into the Caspian Sea ecosystem is much higher than their rate of removal (e.g., burial rate). Of course, given the migratory lifestyle of Caspian seals, it is not possible to identify the exact route and location of PAH contamination of Caspian seals (Anyakora et al., 2005).

No ecotoxicological risk assessments have been conducted to derive the toxic effect concentration and/or safe level thresholds of PAHs on Caspian seals. PAH accumulation in tissues could have negative effects on the health status of this species, even at very low concentrations. Appropriate strategies for limitation of PAH entrance into the Caspian Sea should be considered in countries bordering the Caspian Sea. Further studies are needed to determine pathogenic and lethal doses of PAHs in Caspian seals.

Acknowledgments

This study was supported by Gorgan University of Agricultural Sciences and Natural Resources. The author thanks Dr. James Norman Milles for edition of the earlier manuscript that greatly

improved it. We also show our gratitude to Seyyed Reza Kahleghi for her help in obtaining some sampled Caspian seals.

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Table 1: PAHs concentration (ppb w.w.) in the tissues of Caspian seals based on age and sex.

Sample number	1	2	3	4	5	6	7	8	9	10	Average
Sex	F	F	M	F	F	M	M	M	M	M	
Age	26	17	16	11	19	9	10	20	6	26	
A(BT)	0	56.93	82.44	95.86	44.22	194.55	174.69	0	199.68	0	84.83±79.86
A(LT)	0	6.68	7.34	8.56	6.35	10.42	10.05	7.15	14.27	0	7.08±4.40
A(KT)	0	12.04	12.49	12.24	9.33	17.93	13.06	12.01	27.30	10.24	11.64±7.91
PH(BT)	0	0	0	47.93	0	87.34	27.48	0	154.75	0	31.75±52.22
PH(LT)	0	5.83	7.13	8.64	5.71	9.56	8.31	5.72	12.68	0	6.35±3.96
PH(KT)	0	0	0	0	0	0	0	0	0	0	0
N(BT)	0	26.53	0	47.93	0	58.23	28.46	0	89.85	0	25.1±31.57
N(LT)	0	0	0	7.42	0	8.88	0	0	10.49	0	2.67±4.37
N(KT)	0	0	0	4.08	3.90	8.16	3.58	0	8.53	0	2.82±3.40

Anthracene: A, Phenanthrene: PH, Naphthalene: N, L: Liver, K: Kidney, B: Blubber, F: Female, M: Male

409 **Table 2:** Results of ANOVA test on concentration of PAHs (ppb w.w.) in different tissue samples

410

	Blubber	P	Liver	Kidney
411 Anthracene	84.83±79.86**	0.001	7.08±4.40*	11.64±7.91*
412 Phenanthrene	31.75±52.22**	0.001	6.35±3.96*	0b*
413 Naphthalene	25.1±31.57**	0.01	2.67±4.37*	2.82±3.40*

414 * Unsignificant difference, ** Significant difference ($p<0.05$)

415

416 **Table 3:** Results of Student's t-tests between sex of sampled seals and concentration of PAHs (ppb w.w.) in tissue samples

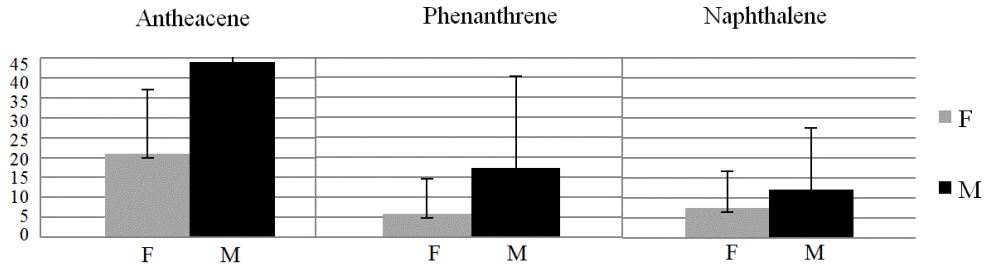
PAHs concentration in liver, kidney and blubber tissues	P	t-value
Concentration of anthracene in blubber tissue	0.00*	0.46
Concentration of anthracene in liver tissue	0.03*	0.08
Concentration of anthracene in kidney tissue	0.04*	3.70
Concentration of phenanthrene in blubber tissue	0.00*	3.58
Concentration of phenanthrene in liver tissue	0.04*	2.28
Concentration of naphthalene in blubber tissue	0.00*	5.95
Concentration of naphthalene in liver tissue	0.01*	3.65
Concentration of naphthalene in kidney tissue	0.03*	8.83

417 * Significant difference ($p<0.05$)

418

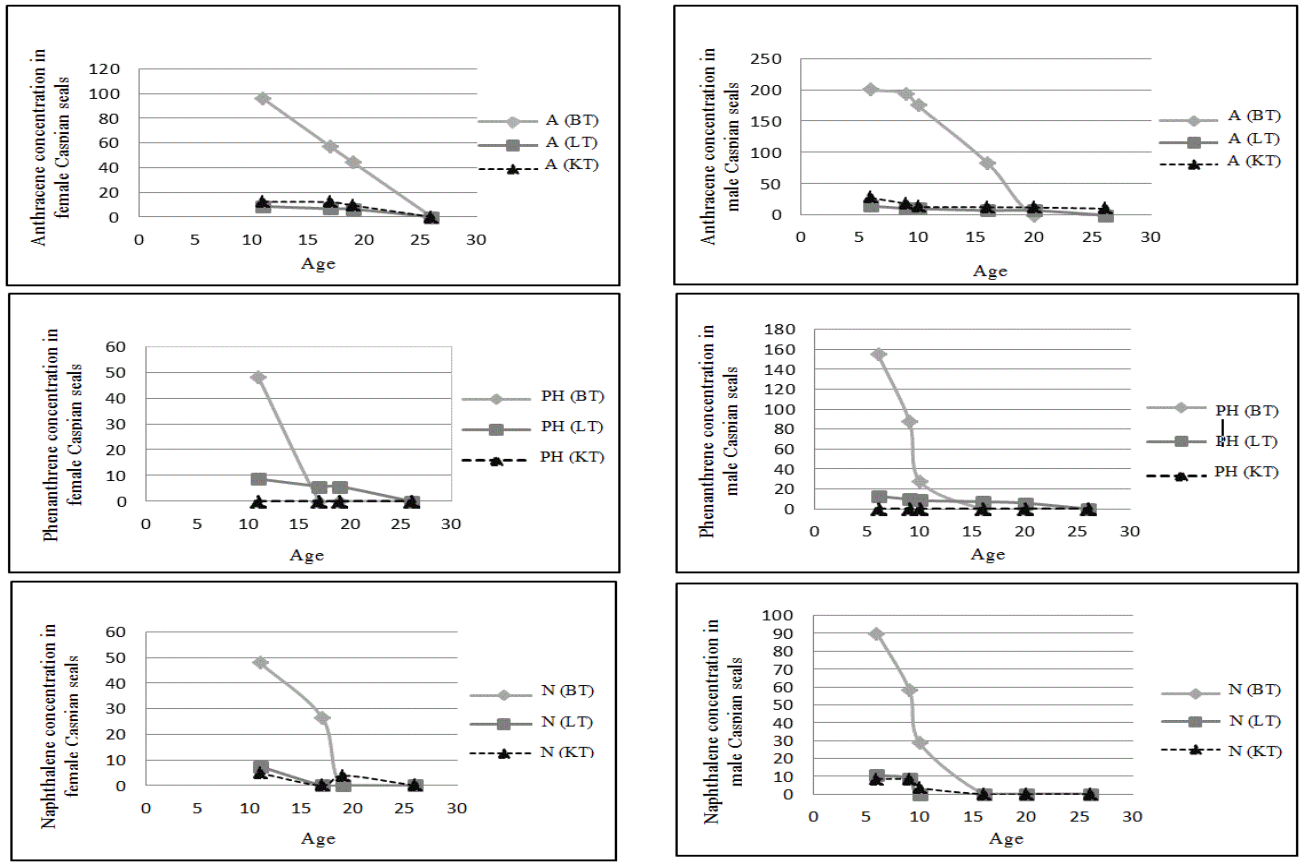
419

Fig 1: Mean (SD) PAHs concentration (ppb w.w.) in male and female Caspian seals



F: female, M: male

Fig 2: Results of liner regression test on relationship between PAHs concentration (ppb w.w.) and age (year) of male and female Caspian seals



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A: anthracene, N: naphthalene, PH: phenanthrene, BT: blubber tissue, LT: liver tissue, KT: kidney tissue