The Biological Effects of Ionising Radiation on Crustaceans: Combining Lab and Field Studies

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Abstract

Amid a renewal of interest in the use of nuclear power and concern regarding past and potential nuclear incidents, assessing the impacts of radiation on the environment has emerged as an area of international scientific and regulatory concern. At present, the impacts of chronic low-dose radiation on non-human organisms are poorly understood. Crustaceans are internationally important model organisms in the field of radioecology and are of commercial and ecological importance. Consequently, this study aimed to adopt a combined laboratory and field approach to assessing the impacts of radiation on crustaceans. Both marine and freshwater crustaceans were exposed to environmentally relevant doses of beta radiation in the laboratory and a range of endpoints including male fertility, DNA damage and development were monitored. In addition, crustacean samples were collected from contaminated environments at Chernobyl and Fukushima and effects on development, reproduction and genetic diversity were assessed. In the laboratory, significant effects on male fertility and sperm DNA damage of the marine crustacean, *Echinogammarus marinus*, were recorded at doses of 1 mGy/d, with some evidence for knock-on impacts on female reproduction at lower doses of 0.1 mGy/d. No significant effects on male fertility were recorded in the freshwater crustacean, *Gammarus pulex*. No significant effects on reproduction, development or genetic diversity of crustaceans *Asellus aqauticus* and *Eriocheir japonica* (development only) were recorded at Chernobyl and Fukushima respectively. Laboratory studies suggest effects at dose levels below those proposed for environmental radioprotection, though the population-level consequences of these effects are unclear. Conversely, field studies of crustaceans at both Chernobyl and Fukushima found no significant effects of chronic radiation exposure. Based on these results, laboratory studies may provide overly conservative risk assessments of radiation impacts in the environment. These findings challenge current understanding in radioecology and will aid in assessing the risk posed by radiation to aquatic biota and the management of contaminated environments.

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Declaration: Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abbreviations List

Abbreviation	**Definition**
µGy	Microgray
AFLP	Amplified Fragment Length Polymorphism
AFSW	Artificial Seawater
AFW	Artificial Freshwater
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCF	Biological Concentration Factor
bp	Base pairs
Bq	Becquerel
CFA	Composite Fluctuating Asymmetry
CR	Concentration Ratio
CNPP	Chernobyl Nuclear Power Plant
DA	Directional Asymmetry
DCC	Dose Conversion Coefficient
DCRL	Derived Consideration Reference Level
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double Strand Break
EC	European Commission
ERICA	Environmental Risk from Ionising Contaminants: Assessment and Management
F0	Parental Generation
F1	First Filial Generation
F2	Second Filial Generation
FA	Fluctuating Asymmetry
FAO	Food and Agriculture Organisation
FDNPP	Fukushima Dai-ichi Nuclear Power Plant
FREDERICA	FASSET Radiation Effects Database
FZ	Funazawa
GBS	Genotyping-by-Sequencing
GLM	General Linear Model
Gy	Gray
H_e	Expected Heterozygosity
H_o	Observed Heterozygosity
HSI	Hepatosomatic Index
HTO	Tritiated Water
IAEA	International Atomic Energy Agency
IBD	Isolation-by-Distance
ICRP	International Commission on Radiological Protection
IER	Institute of Environmental Radioactivity
IMS	Institute of Marine Sciences
IR	Ionising Radiation
LD_{50}	Lethal Dose (for 50% of test organisms)
ME	Measurement Error

MEXT	Ministry of Education, Culture, Sports, Science and Technology, Japan
MHLW	Ministry of Health, Labor and Welfare, Japan
MMS	Methyl Methanesulfonate
mSv	Millisievert
MTT	Methyl Thiazol-diphenyl-tetrazolium bromide
MDS	Multi-Dimensional Scaling
NCRP	National Council on Radiation Protection and Measurements
NR	Niida River
NHEJ	Non-homologous end joining
NPA	National Police Agency of Japan
OECD	Organisation for Economic Co-operation and Development
PBq	Petabecquerel
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PROTECT	Protection of the Environment from Ionising Radiation in a Regulatory Context
RAP	Reference Animals and Plants
RAPD	Random Amplified Polymorphic DNA
RBE	Relative Biological Effectiveness
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism F
ROS	Reactive Oxygen Species
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SPA	Special Protection Area
SSSI	Site of Special Scientific Interest
Sv	Sievert
TASSEL	Trait Analysis by aSSociation, Evolution and Linkage
TLD	Thermoluminescent Dosimeter
TUE	Transuranium Elements
TBTO	Tributyltin Oxide
UN	United Nations
UNEAK	Universal Network-Enabled Analysis Kit
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
UR	Matsukawa-ura
US	Ukedo River
US DOE	United States Department of Energy
USSR	Union of Soviet Socialist Republics

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Publications

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Conferences

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Chapter 1

Introduction

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1.1 Sources & Effects of Ionising Radiation

1.1.1 What is Ionising Radiation?

All forms of ionising radiation (IR) are caused by unstable atoms which release energy in the form of particles and/or electromagnetic radiation. The main forms of ionising radiation are alpha, beta and gamma radiations, whilst x-rays are also a form of IR. Alpha particles are large, heavy and positively charged particles that are not able to penetrate through skin and are generally stopped by a few centimetres of air. By contrast, beta particles are small and may be positively (e.g. positrons) or negatively charged, are fast moving and capable of penetrating the skin up to 1 – 2 cm depending on their energy. Both gamma radiation and x-rays are high energy electromagnetic waves that have no mass or charge and can easily penetrate human skin. The type of radiation and the speed at which it is produced varies between radioactive substances. Owing to decay, the amount of a radioactive material is in continuous decline, and the time taken for half of a given radionuclide to undergo decline is known as its half-life. Amounts of radioactivity are typically measured in a unit known as Becquerels (Bq), which corresponds to the number of transformations per second. One Bq is defined as the activity of a given radioactive substance in which, on average, one nucleus decays per second.

Organisms are typically exposed to radiation through two pathways: a) external irradiation wherein the source of radiation is outside of the body in environmental media such as water, air or soil and b) internal irradiation where the radioactive material is within the organism following inhalation, ingestion or absorption. Radiation doses to organisms are typically measured in Grays (Gy), with one Gray being equal to one Joule of radiation energy per kilogram of biological tissue. Since different types of radiation interact with biological material in different ways, equal doses may not have the same biological effects. For example, 1 Gy from alpha radiation would be more harmful than 1 Gy of beta radiation, owing to the slower and heavier alpha particle losing energy more densely along its path (International Atomic Energy Agency [IAEA, 2004]). This difference in damage from different radiations is termed relative biological effectiveness (RBE) which relates the ratio of biological effectiveness of a given type of IR to another given the same amount of absorbed energy. To account for these differences, the most commonly used unit in human radioprotection is the Sievert (Sv), or equivalent dose. This unit takes into account the differences in RBE by multiplying the absorbed dose by a factor which considers the potential to cause biological harm. For alpha particles this weighting factor is 20, therefore 1 Gy of absorbed alpha radiation would lead to an equivalent dose of 20 Sv. Conversely, for beta, gamma and x-rays the weighting factor is 1, so the absorbed and equivalent doses are assumed to be numerically equal.

1.1.2 Sources of Radioactivity in the Environment

All organisms are continually exposed to ionising radiation from natural sources. Understanding background exposure to radiation is fundamental to contextualising doses received by organisms from anthropogenic sources (Smith & Beresford, 2005). Natural radiation sources include cosmic radiation originating from the sun and outside the solar system, gamma radiation from radionuclides within the earth’s crust, and radon, a decay product of naturally occurring uranium. Radon gas is typically the most significant source of natural exposure (see Table 1.1), and can present health problems where exposure levels are particularly high (Darby et al., 2005). This is due to the attachment of decay products of radon to fine particles in the air and the irradiation of lung tissue following inhalation (IAEA, 2004). Internal irradiation arises from natural radionuclides in food, water and air. Exposure to man-made sources of radiation is dominated by medical applications, usually in diagnostic procedures. In the UK, average annual radiation exposures are 2.7 mSv (millisievert) per person, with 84% of the dose arising from natural sources and the remaining 16% from artificial sources (Public Health England, 2013).

The majority of anthropogenic radiation in the environment is derived from the following sources: nuclear weapons testing, discharges from nuclear facilities and accidental releases of radioactivity (United Nations Scientific Committee on the Effects of Atomic

Radiation [UNSCEAR], 2000). The testing of nuclear weapons took place from 1945 – 1980 and whilst radioactivity levels were generally low, this practice was the most significant source of exposure of the global population to man-made radionuclides in the environment (UNSCEAR, 2000). Of most significance are two radionuclides, Caesium-137 (^{137}Cs) and Strontium-90 (^{90}Sr), which have long half-lives of 30.2 and 28.8 years respectively and are the major contributor to worldwide doses from man-made radionuclides at present (IAEA, 1995). Doses from weapon-derived Carbon-14 (C^{14}) are expected to be more significant over long time scales however, owing to its long half-life (5730 years) despite much lower individual doses than ^{137}Cs and ^{90}Sr (Prăvălie, 2014). ^{137}Cs and ^{90}Sr can still be detected in rainfall, seawater and soils as a consequence of weapons testing owing to the transfer of radioactive materials to the upper stratosphere and the long timeframe over which these materials return to earth.

Permitted discharges from nuclear facilities are another source of man-made radionuclides in the environment. Such releases are of greatest significance in the aquatic environment, which is an important sink for radionuclides. This is due to atmospheric deposition of fallout being two times higher in ocean ecosystems as compared to terrestrial systems (Burton, 1975), and the majority of waste depositions from nuclear facilities being in liquid form. For example, the Sellafield nuclear spent fuel reprocessing site located in Cumbria, United Kingdom, generated a liquid radioactive effluent of 6.649×10^5 GBq (1×10^9 Bq) beta and gamma emitters (excluding tritium) over a four year period from 1995 – 1999 (European Commission [EC], 2001). Radionuclide contamination from the Sellafield site is detectable in areas of the North Atlantic and the Arctic oceans, representing a significant source of radionuclide contamination (Aarkrog, 2003)

Radionuclides have been inadvertently released into the environment as a consequence of major accidents such as Chernobyl and Fukushima. These accidents will be discussed more comprehensively in Section 1.2. The Chernobyl accident released a total of 78 PBq (1×10^{15} Bq) of caesium (^{134}Cs and ^{137}Cs) into the environment, of which 40 PBq (petabecquerel) was deposited on former USSR (Union of Soviet Socialist Republics) territory. Estimates of the overall input of ^{137}Cs to the world's oceans as a consequence of the Chernobyl accident are 15 – 20 PBq (Aarkrog et al., 2003). By contrast, the accident at the Fukushima Dai-Ichi Nuclear Power Plant (FDNPP) caused by the Great East Japan earthquake in March 2011 led to the release of an estimated 7 – 50 PBq of ^{137}Cs into the atmosphere. Estimates for direct releases to the marine environment range from 1.5 – 5 PBq (IAEA, 2015).

Table 1.1. – Average annual doses to the world population from all sources of radiation. From IAEA (2004).

Source	Dose (mSv)
Natural Sources	
Cosmic Radiation	0.4
Gamma Rays	0.5
Internal	0.3
Radon	1.2
Artificial Sources	
Medical	0.4
Atmospheric Nuclear Testing	0.005
Chernobyl	0.002
Nuclear Power	0.0002

1.1.3 Ionising Radiation and the Environment

Previous guidelines for environmental radioprotection were based upon permitted dose limits for humans on the assumption that if human radioprotection is adequate, non-human organisms would also likely be protected (International Commission on Radiological Protection [ICRP], 1977). However, this does not apply to environments where dose rates exceed permitted limits for humans but are occupied by non-human organisms. Furthermore, this system is distinct from other anthropogenic contaminants wherein protection frameworks and concepts for the environment (e.g. the ecological risk assessment) are well developed (Bréchignac, 2003). Consequently, the need for environmental radioprotection has now been recognised amongst scientific and regulatory communities (IAEA, 2003; ICRP, 2007a). Many countries and regulatory bodies have subsequently established systems and appropriate benchmarks for protection of the environment (see Table 1.2).

The ICRP has established a committee (Committee number 5) focusing on environmental radioprotection, the overarching aim of which is ‘preventing and reducing the frequency of deleterious radiation effects to a level where they would have negligible impact on the maintenance of biological diversity, the conservation of species, or the health and status of natural habitats, communities and ecosystems’ (ICRP, 2007b). A range of different dose rate values have been proposed below which no observable effects on terrestrial and aquatic populations are likely to be observed (see Table 1.2). A generic screening value of 10 µGy/hr (micrograys per hour) has been proposed enabling regulators to identify scenarios of least concern with a high degree of confidence (Howard et al., 2010). This value was the outcome of the EC PROTECT (Protection of the Environment from Ionising Radiation in a Regulatory Context) project following rigorous review of the available biological effect data and consideration of relevant endpoints (Howard et al., 2010). Whilst invaluable for providing a conservative value applicable across a range of scenarios, a species-specific approach may be necessary to ensure protection of the more radiosensitive organisms. Although radiosensitivity varies greatly within taxa (Harrison & Anderson, 1996), it is generally accepted that radiosensitivity increases with the degree of biological complexity (Copplestone et al., 2001). Organisms such as birds, trees and mammals are believed to be more radiosensitive than invertebrates (Hinton et al., 2007). Consequently, in certain circumstances the 10 µGy/hr value may prove to be too conservative, leading to in depth assessments which may be unnecessary. The ICRP have developed the concept of reference animals and plants (RAPs), which involves using a number of different animals and plants as a systematic basis for relating exposure to dose and dose to effects (ICRP, 2007b, see Table 1.2). This approach acknowledges the impracticalities of assessing the environment in its entirety, and instead aims to focus on groups of organisms that are typical of major environments and for which adequate biological data is present. Data derived from these organisms will be used to form a judgement on the probability and severity of effects in a given environment. To support the implementation of the RAP concept in environmental exposure scenarios, the ICRP established derived consideration reference levels (DCRLs). DCRLs are defined as 'a band of dose rates within which there is likely to be some chance of deleterious effects (ICRP, 2009), which the commission recommends are used as criteria in mitigating environmental exposures (ICRP, 2015).

This approach has been adopted by agencies such as the IAEA in monitoring areas impacted by radionuclides. For example, dose rates to crab, flatfish and brown seaweed were calculated in areas impacted by the accident at the Fukushima Dai-ichi Nuclear Power Plant (FDNPP) in March 2011 (IAEA, 2015). Although in the initial phase after the accident dose rates to certain RAPs (e.g. brown seaweed) exceeded levels within which deleterious effects may be expected, monitoring of RAPs allowed the agency to conclude that no significant long-term impacts on populations or ecosystems were expected (IAEA, 2015). This example gives an indication of the current status of environmental radioprotection and its implementation in monitoring and mitigating the impacts of releases of radioactivity on the environment.

Table 1.2 - Numerical benchmark values in µGy/hr proposed by a number of different organisations and directives for the protection of populations of a range of biota. US DOE = United States Department of Energy. NCRP = National Council on Radiation Protection and Measurements. IAEA = International Atomic Energy Agency. - = No data provided. Adapted from Andersson et al., (2009). Reference organisms are written in bold.

	Dose Level (µGy/hr)								
	US DOE (1990)	NCRP (1990)	IAEA (1992)	Environment Canada (2003)	FASSET (2003) (Larsson, 2004)	ERICA (2007) (Beresford, 2007)	ICRP (2008)	UNSCEAR (2008)	PROTECT (2009) (Andersson, 2008)
Freshwater Organisms	400	400	400	-	100	10	-	400	10
Algae	-	-	-	100	-	-	-	-	-
Macrophytes	-	-	-	100	-	-	-	-	-
Benthic Invertebrates	-	-	-	200	-	-	-	-	-
Fish	-	-	-	20	-	-	-	-	-
Trout	-	-	-	-	-	-	40-400	-	-
Frog	-	-	-	-	-	-	4 - 40	-	-
Marine Organisms	400	400	-	-	100	10	-	400	-
Marine Mammals	-	-	-	-	-	-	-	-	-
Deep Ocean Organisms	-	-	1000	-	-	10	-	-	-
Crab	-	-	-	-	-	-	400-4000	-	-
Flatfish	-	-	-	-	-	-	40-400	-	-
Brown Seaweed		-	-	-	-	-	40-400	-	-
Terrestrial Organisms	-	-	-	100	100	10	-	100	10
Plants	-	-	400	-	-	-	-	-	-
Pine Tree	-	-	-	-	-	-	4-40	-	-
Wild Grass	-	-	-	-	-	-	40-400	-	-
Animals	-	-	40	-	-	-	-	-	-
Invertebrates	-	-	-	200	-	-	-	-	-
Bee	-	-	-	-	-	-	400-4000	-	-
Earthworm	-	-	-	-	-	-	400-4000	-	-
Mammals	-	-	-	100	-	-	-	-	-
Deer	-	-	-	-	-	-	4-40	-	-
Rat	-	-	-	-	-	-	4-40	-	-
Birds	-	-	-	-	-	-	4-40	-	-
Duck	-	-	-	-	-	-	4-40	-	-

1.2 The Chernobyl & Fukushima Accidents

1.2.1 Sequence of Events and Consequences

i) Chernobyl Accident

On the 26th April 1986, reactor four of the Chernobyl nuclear power plant (CNPP) exploded following a planned test of the back-up electrical system. A number of factors contributed toward the explosion including a design flaw in the reactor itself, unstable low-power conditions at the power plant and the deactivation of certain safety systems by plant operators prior to the test (UNSCEAR, 2000). Despite the efforts of helicopter pilots and emergency workers, a graphite fire burned for ten days, dispersing vast amounts of radioactivity into the environment (Smith & Beresford, 2005). In the initial phase following the accident, emergency workers and power plant operators were exposed to very high doses of radiation. A total of 134 suffered from acute radiation sickness, with 28 of these dying in the following months after the accident (UNSCEAR, 2000).

Areas of the Ukraine, Belarus, Russia and Western Europe were contaminated by radioactive substances, with the deposition of radionuclides mainly governed by precipitation occurring during the passage of the radioactive plume. This led to a complex and heterogeneous pattern of contamination throughout affected regions. In 1986 an initial 115,000 people inhabiting impacted areas were evacuated, rising to a total number of 220,000 across Belarus, Russia and the Ukraine after 1986 (UNSCEAR, 2000). The relocation of this number people led to serious psychological and socioeconomic consequences. The comprehensive 2005 report of the Chernobyl forum stated that the effect of the accident on mental health was ‘the largest public health problem unleashed by the accident to date’ (Balonov, 2007).

ii) Fukushima Accident

The Great East Japan Earthquake of magnitude 9.0 occurred on the 11th March 2011 off the eastern coast of Japan. The earthquake triggered a series of large tsunami waves that had a devastating impact, causing greater than 15,000 deaths and destruction of residential and industrial areas (National Police Agency of Japan [NPA], 2015). The tsunami overwhelmed the protective barriers of the Fukushima-Daiichi Nuclear Power Plant (FDNPP), causing loss of on-site power, compounding the damage inflicted to the off-site power systems by the earthquake (IAEA, 2015). Both on and off-site power was lost (known as a station blackout event) in five of the six nuclear reactors at the FDNPP, lasting for 14 days in Units 3 & 4 and 9 days in Units 1 and 2. The extended loss of power led to reactor damage from the overheating and melting of fuel in Units 1-3, leading to the release of vast amounts of radionuclides. Estimated releases of ^{137}Cs to the atmospheric source term range from 7 – 20 PBq (IAEA, 2015). Radionuclide releases from Fukushima are estimated to be approximately one tenth of those from the Chernobyl accident (IAEA, 2015). Workers at the site of FDNPP and residents of the surrounding area were exposed to radiation, along with radiological contamination of the environment. Evacuation of more than 100,000 people was conducted within a 20 km radius of the site due to radioactive releases to the environment. At present no deaths have been directly attributed to radiation exposure at Fukushima, nor are discernible stochastic effects such as elevated incidence of leukaemia or solid cancers expected to be detected epidemiologically (UNSCEAR, 2016). However, the evacuation procedure of hospital inpatients and elderly citizens and lack of immediate medical support caused substantial fatalities (Tanigawa et al., 2012; Hasegawa et al., 2016).

1.2.2 Initial Environmental Effects

i) Chernobyl

Approximately 40% of the 30-kilometre zone around the CNPP was forested, comprising mostly of coniferous trees (Kryshev et al., 2005). Forested areas along the westward moving plume of contamination received large radiation doses immediately after the accident. Acute doses of 80 – 100 Gy to coniferous trees led to mortality in an approximately 4 km^2 zone of pines. Sub-lethal effects including morphological changes and death of most growth points were recorded in a larger 38 km^2 area (see Smith & Beresford, 2005 and Beresford et al., 2016 for summary). Initial effects were more subtle in herbaceous plants, with genetic mutations observed in a range of species but non-significant alterations to seed viability parameters in the majority of the 30 km exclusion zone (Abramov et al., 1992; Kryshev et al., 2005; Taskaev et al., 1988). Owing to the concentration of fallout in the upper soil layer, soil fauna in pine forests received considerable radiation doses and declines in the number of soil-dwelling mites and sexually immature invertebrates were recorded (Krivolutsky et al., 1990, 1994). No effects were recorded in the structure and population dynamics of above-ground insects (Smith & Beresford, 2005). In mammalian organisms, a population reduction in the number of mice was recorded immediately after the accident, though evidence suggests population sizes increased afterward owing to the removal of humans and abundant food supply (Testov & Takaev, 1990).

Limited studies focused on large mammals such as horses and cattle abandoned within the evacuated area after the accident. For cattle, estimated doses to the thyroid of 150 – 200 Sv from the short lived Iodine-131 (^{131}I) led to mortality or hypothyroidism in surviving animals (IAEA, 1991). Further studies conducted by the Food and Agriculture Organisation (FAO) of the United Nations (UN) reported that effects were largely limited to the first generation of these organisms (IAEA, 1991). Much of the available data for large mammals in the initial phase after the accident relates to uptake and the environmental transfer of radionuclides as opposed to effects. Highest concentrations of ^{137}Cs and ^{90}Sr were found in wild boar and roe deer, being 270 and 730 kBq kg^{-1} respectively for muscle and bone (Gaschak et al., 2003). Doses and accumulation of radionuclides by aquatic organisms will be discussed in more detail in Section 1.2.3.

ii) Fukushima

Greater than 80% of the atmospherically released radionuclides from the Fukushima accident were deposited offshore in the neighbouring Pacific Ocean (Steinhauser et al., 2014) leading to radiation exposure of marine organisms. Amongst the marine biota, brown seaweed received the highest doses of 70 mGy/d over the initial period of 0 – 30 days following the accident (IAEA, 2015). Though such dose rates are expected to cause deleterious effects in brown seaweed, these applied only for organisms within 30 metres of the release point and for the immediate aftermath of the accident. Therefore, population-level effects on brown seaweed were not expected as a consequence of the accident. Dose rates for other marine organisms were significantly lower, with a maximum of 3.4 mGy/d estimated for flatfish (Vives i Batlle et al., 2014). According to DCRL values proposed for reference organisms (see Section 1.1.3 above for further detail), the available data suggests dose rates to marine biota in the initial phase after the accident would not have induced significant effects. However, at present no studies have conducted direct observations of potential individual-level effects of Fukushima-derived radiation on marine organisms.

Within terrestrial ecosystems, plants received the highest dose rates immediately following the accident. Estimated total accumulated doses to pine trees for the first 30 days were 0.6 Gy (IAEA, 2015). Based on data obtained from forested areas following the Chernobyl accident, severe sub-lethal effects such as partial death and meristem destruction are anticipated at accumulated doses of 10 – 20 Gy. Therefore, the large scale damage and mortality to pines observed at Chernobyl (see Sect 1.2.2i) was not expected following the Fukushima accident (Yoschenko et al., 2017). Dose rates for other terrestrial biota typically fell within or below the DCRLs proposed by the ICRP, meaning severe ecological effects were considered highly unlikely (IAEA, 2015). However, a number of field studies of organisms have reported negative effects on biota in the exclusion zone around the FDNPP. For example, Møller et al., (2012) reported a reduced abundance of 14 bird species at Fukushima based on census counts in July 2011. Similarly, a one year study of Japanese monkeys collected from Fukushima from April 2012 recorded lower white blood cell counts as compared to a reference population (Ochiai et al., 2014). However, both of the aforementioned studies have been subject to criticism relating to experimental design and statistical analysis (Beresford et al., 2012). Further studies are therefore necessary to determine the chronic effects of Fukushima radiation on non-human organisms.

1.2.3 Initial Impacts on Aquatic Systems

i) Chernobyl

The Chernobyl nuclear power plant is located next to the Pripyat river, a component of Dnieper-river reservoir system, which is one of the largest surface water systems in Europe (Smith & Beresford, 2005). Owing to the potential for transfer of contamination to the Kiev reservoir, a major source of drinking water for the city, contamination of aquatic systems became an important issue immediately after the accident. A range of lakes and reservoirs around Europe were contaminated with radioactive substances, showing relatively rapid decline in activity concentrations in many systems owing to significant inflows and outflows of water and the subsequent ‘flushing’ effect. In the immediate vicinity of Chernobyl however, many ‘closed’ lake systems with limited water movement retained high activity concentrations of radionuclides, which in turn led to elevated radionuclide concentrations in aquatic biota (Smith & Beresford, 2005). Activity concentrations of radionuclides in such closed lake systems in the vicinity of the CNPP were as high as 74 and 370 Bq l^{-1} for ^{137}Cs and ^{90}Sr respectively in 1991 (Vakulovsky et al., 1994). In the initial days following the accident, maximum dose rates from water and bottom sediments of 2-3 and 100-200 mGy/d were calculated (Kryshev et al., 2005). Dose rates decreased by several orders of magnitude within two years owing to decay of short lived radionuclides and settling of radioactive substances to the bottom. Bioaccumulation of radiocaesium in freshwater fish and activity concentrations above permissible limits for consumption is an ongoing concern in many areas impacted by the accident (e.g. Finland, Rask et al., 2012).

ii) Fukushima

Following the Fukushima accident, radioactivity entered the marine environment via several pathways. Due to weather conditions at the time of the accident and the proximity of the

FDNPP to the Pacific Ocean, greater than 80% of the radionuclides released to the atmosphere were deposited on to the ocean surface (Steinhauser et al., 2014). The exact amounts of radioactivity that were initially deposited remain uncertain, as measurements were not conducted during the initial phase following the accident (IAEA, 2015). Radionuclides were also directly deposited into the Pacific from contaminated water. Water was originally used to provide cooling for the damaged reactors, leading to releases of large volumes of contaminated effluent. In addition, structural damage to the FDNPP site led to leaks and subsequent discharge to the ocean. Intentional discharge of relatively low-level wastewater was also performed as a preventive measure in April 2011. This was conducted to prevent a leak of highly contaminated wastewater which was accumulating within one of the damaged units. Low level contaminated waste within the radioactive waste treatment facility and sub-drains of other units was discharged to be replaced with the highly contaminated waste (IAEA, 2015). Estimates for the amount of radionuclides directly discharged into the ocean vary from 1 – 6 PBq (IAEA, 2015).

Fukushima prefecture has more than 3700 freshwater ponds as well as several major river systems, ensuring that radionuclide contamination of freshwater systems is a major issue (Wakiyama et al., 2017). Following the accident, distribution and fluxes of radiocaesium in major catchments close to the FDNPP have been studied extensively (Nagao et al., 2015; Yamashiki et al., 2014; Yoshimura et al., 2015). Several fundamental differences exist as compared to Chernobyl in terms of contamination of the aquatic environment (see Konoplev et al., 2016 for a review). For example, differences in annual precipitation and the mountainous terrain at Fukushima means that wash-off from contaminated catchments is much greater at Fukushima, reaching 5% per year in certain areas compared to Chernobyl where 1% wash-off per year was never reached. Radiocaesium accumulation in marine and freshwater fishes has been studied extensively in the context of food safety (see Wada et al., 2016a, 2016b for summary). Marine food products have shown a decreasing trend in the years following the accident, with only 0.05% of samples exceeding the conservative

Japanese regulatory limit for consumption of 100 Bq kg^{-1} in 2015 (Wada et al., 2016a). Comparatively, freshwater fish have shown a slower decreasing trend with higher levels of contamination, though radiocaesium activity concentrations are generally an order of magnitude lower than freshwater fish at Chernobyl (Wada et al., 2016b). The complex and heterogeneous environment at Fukushima ensures that contamination of aquatic biota will be an ongoing issue in years to come.

1.3 Effects of Radiation on Aquatic Invertebrates

Invertebrates constitute around 90% of extant life and are routinely used in toxicology testing of potential harmful compounds as an alternative to mammalian models (Guilhermino et al., 2000). The freshwater cladoceran invertebrate, *Daphnia magna*, has gained prominence as a model organism and is commonly used in risk assessments of chemicals in the environment by governmental organisations (e.g. The Organisation for Economic Co-operation and Development [OECD], 2004, 2012). The effects of IR on aquatic invertebrates have been comprehensively reviewed by Dallas et al., (2012). The authors highlighted the lack of data available for aquatic invertebrates compared with higher organisms such as fish and mammals, and found that the dose levels at which effects occur in aquatic invertebrates varies drastically. Furthermore, a significant knowledge gap was identified regarding radiation effects at environmentally relevant dose rates.

Owing to the importance of crustaceans as reference organisms in radioecology (see Section 1.13), the present study aimed to adopt a phyla-specific approach to critically assess the literature of the impacts of radiation on crustaceans. This was conducted to prioritise research needs and support the development of robust, applicable ecological benchmark values for environmental radioprotection (Fuller et al., 2015).

1.4 Review of the Effects of Radiation on Crustaceans

1.4.1 Crustaceans as Model Organisms

Members of the subphylum Crustacea are the dominant components of aquatic ecosystems globally, comprising greater than 66,000 species (LeBlanc, 2007). These organisms provide an array of commercial and ecological services and are used both directly for human consumption and as a food source for other commercially important species (Benzie, 2009). Crustaceans have gained prominence as model organisms in ecotoxicology and evolutionary ecology owing to the relative ease by which they can be cultured in laboratory environments and increasing knowledge of crustacean genomics and biological systems.

Due to their ubiquity in aquatic environments and well characterized biology, a marine crustacean of the family Cancridae has been selected as one of the ICRPs reference animals and plants (ICRP, 2007a). The concept of ‘RAPs’ has been explained in Sect. 1.1.3 and highlights the importance of understanding radiation effects on crustaceans for both members of the scientific and regulatory communities. Radiation effects on the four ‘umbrella endpoints’ of mutation, morbidity, mortality and reproduction (Copplestone et al., 2008) will be considered in the below review.

1.4.2 Effects of Radiation on Mutation

A mutation is defined hereafter as “a change in the chromosome or genes of a cell which may affect the structure and development of the resultant offspring” (Copplestone et al., 2008). Despite evidence suggesting the clastogenic (capacity to cause chromosomal aberrations) and mutagenic (capacity to induce genetic mutations) potential of ionising radiation observed in a range of organisms including humans (Lucas et al., 1992), fish (Anbumani & Mohankumar, 2012; Kligerman et al.,1975) and molluscs (AlAmri et al., 2012), there is a paucity of information within the literature regarding crustaceans. For example, the FREDERICA (FASSET Radiation Effects Database) database containing over 30,000 data entries collated from several international radiation effects directives contains no data regarding mutation in crustacean species over chronic dose ranges of 0 - > 10000 µGy/hr.

Field studies have suggested that mutation may be a sensitive endpoint of radiation-induced effects in crustacean species. For example Florou et al., (2004) assessed chromosomal aberrations in microfauna collected from geothermal spring areas on the island of Ikaria, Greece where maximum dose rates of natural gamma emitters in sediments were 9.6 mGy yr (~ 0.001 mGy/hr). These values are substantially elevated above the reported mean of 0.07 mGy yr (~ 0.008 µGy/hr) for coastal sediments in Greece (Florou & Kritidis, 1992). An elevated level of cells displaying chromosome aberrations (3.8%) was recorded in populations of the amphipod crustacean *Melita palmata* collected from these areas compared with control sites (1.5 – 1.7%). However, the cytogenetic response may have been due to the complex environmental conditions typical of geothermal springs (e.g. Duggan et al., 2007) as opposed to the direct effects of ionising radiation. This highlights the inherent difficulties in field radioecology studies (Salbu, 2009) and the importance of quantifying the individual contribution of stressors in environments where abiotic pressures may act synergistically (Dallas et al., 2012). This study represents the only study of natural crustacean populations using mutation as an endpoint. Laboratory studies assessing radiation-induced mutations in crustaceans typically involve acute high doses that are unrepresentative of environmental exposures. Such studies have, however, demonstrated the ability of ionising radiation to induce chromosomal aberrations. Tsytsugina (1998) exposed embryos of two crustacean species, *Idotea baltica* and *Gammarus olivii* to doses of 0.5 to 5 Gy from a range of radionuclides and recorded a concomitant increase in chromosomal aberrations with radiation dose.

Recent approaches to assessing radiation-induced genotoxicity in aquatic invertebrates have involved monitoring levels of the expression of genes that are involved in DNA damage repair pathways (AlAmri et al., 2012; Han et al., 2014a; Han et al., 2014b; Won & Lee, 2014). For example, Han et al., (2014a) exposed cultures of the intertidal copepod, *Tigriopus japonicus* to gamma radiation from ^{137}Cs and monitored mRNA expression of three DNA repair genes: *Ku70 (Xrcc6), Ku80 (Xrcc5)* and *DNA-PK*. These three genes are integral to the non-homologous end joining (NHEJ) DNA repair pathway involved in the detection and repair of radiation-induced double strand breaks (DSBs, Mahaney et al., 2009). Expression of the three genes was significantly elevated with respect to controls in 200 Gy exposed organisms, suggesting induction of DSBs at these dose levels (Han et al., 2014a). The potential of this approach as a biomarker for genotoxicity in crustacean species was emphasised by Won & Lee (2014) who reported a dose dependent increase in mRNA expression of these genes in another copepod species, *Paracyclopina nana*. A recent study by Sarapultseva et al., (2017) adopted a novel approach in assessing cytotoxicity of radiation exposure in *D. magna* using the methyl thiazol-diphenyl-tetrazolium bromide (MTT) cell viability assay. The authors found that following acute doses of 0.1, 1 and 10 Gy from ^{60}Co, a cytotoxic effect was observed both in exposed organisms and non-exposed offspring.

Radiation induced molecular level effects have also been recorded at lower, environmentally relevant doses. For example, Parisot et al., (2015) reported significant DNA alterations in *Daphnia magna* following exposure to ^{137}Cs at doses of 0.007 mGy/hr using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) methods. Molecular level responses were evident at both lower doses and shorter exposure durations than other endpoints including mortality, morbidity and perturbations to reproduction.

1.4.3 Radiation Impacts on Morbidity

Morbidity can be broadly defined as “A loss of functional capacities generally manifested as reduced fitness, which may render organisms less competitive and more susceptible to other stressors, thus reducing their life span” (Copplestone et al., 2008). Within the crustacean subphylum, a vast array of different endpoints have been used to assess morbidity (see Table 1.3 for summary of studies). Accordingly, this section will be subdivided into effects on growth and respiration and behavioural and histopathological effects.

i) Effects of Radiation on Growth and Respiration in Crustaceans

Alonzo et al., (2006, 2008a) investigated the effects of chronic internal exposure to the alpha emitting radionuclide, ^{241}Americium, on the growth dynamics of *Daphnia magna*. The authors recorded a significantly lower dry mass and body length of irradiated specimens at doses of ~1.5 mGy/hr in parental generation organisms (F0), with significant increases in the severity of effects over generations. For example, individuals of the F2 (second filial generation) generation displayed a 15% reduction in dry mass at doses of 0.3 mGy/hr (Alonzo et al., 2008a). A recent study further underpinned the potential of ionising radiations to perturb growth dynamics in daphnids (Parisot et al., 2015), with reductions of 5 and 13 % in the growth rate of F2 generation daphnids exposed to 4.7 and 35.4 mGy/hr of gamma radiation respectively.

In the previous study (Alonzo et al., 2008a), oxygen consumption of *D. magna* was elevated above controls at all doses, suggesting an increase in metabolic expenditure induced by radiation stress. Exposure of organisms to stressors and adverse conditions may result in reallocation of metabolic energy towards maintenance and lead to reduced energy investment per offspring (Baillieul et al., 2005). This was reflected by a reduced resistance to starvation recorded in neonates derived from 0.02 mGy/hr exposed adult daphnids (Alonzo et al., 2006).

A recent study (Sarapultseva & Gorski, 2013) further suggested deleterious impacts on neonates relating to metabolic perturbations. Following parental exposure to acute gamma doses of 100 and 1000 mGy from Cobalt-60, a ~20% decrease in the mean life span of non-exposed first generation *D. magna* offspring was demonstrated. Recent approaches have considered species-species interactions as morbidity endpoints in radioecology. For example, Nascimento & Bradshaw (2016) studied the effects of gamma radiation from ^{137}Cs on *Daphnia magna* grazing on the phytoplankton, *Raphidocelis subcapitata*. The authors found a dose-dependent decrease in grazing on *R. subcapitata* by *D. magna* exposed to acute doses of 5, 50 and 100 Gy evidenced by lower Carbon incorporation.

ii) Effects of Radiation on Behaviour and Histopathology in Crustaceans

Ionising radiation has been demonstrated to induce behavioural changes in a number of crustacean species including crabs (Engel, 1967), prawns (Stalin et al., 2013a) and crayfish (Rodriguez & Kimeldorf, 1976). The available literature regarding behavioural impacts of radiation involves mostly acute exposures to high doses of radiation (Engel, 1967; Rodriguez & Kimeldorf, 1976), with the magnitude of behavioural changes correlating with dose levels. For example, Engel (1967) assessed the impact of both chronic and acute radiation exposures on the behaviour of the blue crab, *Callinectes sapidus*, a highly aggressive and cannibalistic species (Bushmann, 1999). A reduction in aggressiveness of *Callinectes sapidus* specimens subject to single acute irradiations with ^{60}Co doses from 40 – 640 Gy was observed, whilst higher doses induced a catatonic state. Continuous exposures to lower doses (0.72, 1.64 & 6.53 Gy/d) for 70 days induced cessation of feeding and abnormal behavioural patterns deviating from the normal pugnacious nature of *C. sapidus*, with the extent of behavioural effects relating to dose. Whilst the received dose remains significantly higher than estimates of the highest external doses in freshwater systems immediately after the Chernobyl accident ([4.2 – 8.3 mGy/hr from bottom sediments] Kryshev et al., 2005), the finding that prolonged exposures may perturb behavioural patterns has implications for contaminated areas where radiation levels remain elevated over long time scales. Furthermore, limited data suggests induction of behavioural effects at lower, environmentally relevant doses. Stalin et al., (2013a) demonstrated behavioural changes including alterations to swimming patterns in the giant freshwater prawn, *Macrobrachium rosenbergii*, at acute gamma doses of 3 mGy.

Few studies have considered the impacts of ionising radiation on morphological and histological parameters in crustaceans. Stalin et al., (2013a,b) demonstrated induction of histological and morphological aberrations including swollen and necrotic lamellae in the gill, deformations of the uropod, and discolouration of the abdomen in *M. rosenbergii* over a dose range of 3 - 3000 mGy (Stalin et al., 2013a), with the magnitude of effects relating to dose. Iwasaki (1973) adopted a histological approach to assess gamma radiation-induced effects in oogonia and oocytes of the brine shrimp, *Artemia salina.* A dose-dependent increase in cellular deformations and the number of pyknotic cells (cell degradation characterised by chromatin condensation) was recorded over a high dose range of 250 – 3000 Gy from Cobalt-60. Furthermore, Mothersill et al., (2009) recorded perturbations to cytoplasmic organelles in hematopoietic cultures of *Nephrops norvegicus* at gamma radiation doses of 0.5 Gy. Deformations included abnormal mitochondrial-rough endoplasmic reticulum complexes at 0.5 Gy, progressing to complete disintegration of the cellular cytoplasm at doses of 5 Gy. A decrease in the hepatosomatic index (HSI) of *M. rosenbergii* was also observed as a consequence of radiation exposure (Stalin et al., 2013b) which may provide further evidence that radiation elicits alterations to energy budgets since changes to the HSI may reflect mobilization and utilization of energy reserves (Sánchez-Paz et al., 2007).

Table 1.3 – Summary of morbidity studies in Crustacea. HTO represents Tritiated Water. Acute exposures are defined here as those lasting less than 24 h, with chronic exposures lasting over a period of the organisms life span and greater than 24 h. From Fuller et al., (2015).

Species	Dose Rate/Absorbed Dose	Radiation Source	Exposure Duration	Conclusion	Reference
Pollicipes polymerus	7.9, 62.5 nGy/hr, 6.25 and 62.5 µGy/hr	HTO	Chronic	Altered moulting patterns	Abbott & Mix, (1979)
Daphnia magna	0.02, 0.11 and 0.99 mGy/hr	^{241}Am	Chronic	Reduction in body mass, Increased respiratory demand and Reduction in offspring fitness	Alonzo et al., (2006)
Daphnia magna	0.3, 1.5 and 15 mGy/hr	^{241}Am	Chronic	Increased oxygen consumption, Reduction in body size and mass across generations	Alonzo et al., (2008a)
Artemia salina	100, 200, 400 and 800 Gy	^{60}Co	Acute	Decrease in respiration rate	Angelovic & Engel (1968)
Callinectes sapidus	40,80, 160, 320 and 640 Gy	^{60}Co	Acute	Behavioural changes; reduction in irritability, catatonic state at high doses	Engel, (1967)
Daphnia magna	0.41, 4.2 and 31 mGy/hr	^{137}Cs	Chronic	Decrease in mass-specific respiration rate, Reduction in offspring fitness	Gilbin et al., (2008)
Palaemonetes pugio & Uca pugnax	9.1, 18.2, 45.5, 91.0, 181.9 and 363.9 Gy	^{60}Co	Acute	Alterations to moulting patterns	(Rees, 1962)
Pacifastacus leniusculus trowbridgii	2.8, 5.6, 8.4,11.2 and 16.8 Gy	X-Ray	Acute	Behavioural changes; detection and avoidance of radiation source	Rodriguez & Kimeldorf (1976)
Macrobrachium rosenbergii	3,30,300 and 3000 mGy	^{60}Co	Acute	Behavioural changes, histological aberrations to the gill	Stalin et al., (2013a)
Macrobrachium rosenbergii	3,30,300 and 3000 mGy	^{60}Co	Acute	Morphological deformations, Decreased hepatosomatic index	Stalin et al., (2013b)

1.4.4 Effects of Radiation on Reproduction

Reproductive endpoints are frequently the subject of ecotoxicological and environmental risk assessments studies since perturbations to reproduction may impact upon the long-term survival of a species and hence alter ecosystem dynamics (Anderson & Wild, 1994; Dallas et al., 2012). Many publications have focused on radiation-induced effects on reproductive parameters in aquatic invertebrates, with the reported dose level at which significant effects occur varying by at least two orders of magnitude (Harrison & Anderson, 1996). This variability is exemplified within the crustacean subphylum; for example Alonzo et al., (2008a) recorded a delayed brood production in *D. magna* exposed to 15 mGy/hr over a 23 day period (total dose of 0.345 Gy), however 10 Gy was needed to elicit a delay in the reproduction of the marine copepod, *Paracyclopina nana* (Won & Lee, 2014). Differences in the exposure duration, specific radionuclide and endpoint employed preclude development of a generalised 'dose limit' for reproductive effects in crustacean species.

The available literature within the crustacean subphylum suggests the presence of effects over multiple generations (Alonzo et al., 2008a; Plaire et al., 2013; Massarin et al., 2010; Parisot et al., 2015; Sarapultseva & Gorski, 2013). Alonzo et al., (2008a) and Massarin et al., (2010) recorded an increase in the magnitude of deleterious effects across generations in *Daphnia magna* exposed to chronic alpha irradiation and chronic waterborne uranium exposure respectively, with severe impacts to fitness and reproduction in individuals of the F_2 generation. In contrast, the multigenerational study of Parisot et al., (2015) reported a degree of recovery in F_1 (first filial generation) generation daphnids and a reduced radiosensitivity relative to the parental generation across both lethal and sub-lethal endpoints (mortality and fecundity respectively). This was supported by Sarapultseva et al., (2017) who recorded reduced fecundity in gamma irradiated *Daphnia magna* and non-exposed offspring, however effects were only evident at the highest doses of 10 Gy in second generation offspring.

Studies within the crustacean subphylum are heavily biased toward female reproductive success, with typical endpoints including production of eggs (Won & Lee, 2014), hatchability of eggs (Iwasaki, 1964; Sellars et al., 2005) egg mass (Alonzo et al., 2006, 2008a) and time of hatching (Gilbin et al., 2008). Comparatively, radiation-induced effects on male fertility have been ignored. To the author's knowledge no study has directly recorded the impacts of ionising radiation on male fertility in crustaceans. Sperm are considered sensitive to the influence of xenobiotic stressors including ionising radiation (Fischbein et al.,1997; Lewis & Ford, 2012; Marques et al., 2014). This is attributed to their lack of inherent defence systems such as antioxidant enzymes and DNA repair comparable with other biological systems (Trapp et al., 2015). Experimental evidence in aquatic invertebrates has suggested that reductions in sperm numbers may have subsequent effects at higher levels of biological organisation. For example, Dunn et al. (2006) recorded a 55% reduction in the size of freshwater amphipod (*Gammarus duebeni*) broods after mating with males displaying a sperm count reduction of 56% (Lewis & Ford, 2012). Coupling the ecological relevance of perturbations to sperm parameters with the known sensitivity of sperm, this represents a substantial knowledge gap. Furthermore, to the author's knowledge no study has considered reproductive effects in crustaceans at Chernobyl, despite evidence of radiation-induced reproductive effects in a range of biota including birds (Møller et al., 2005), fish (Belova et al., 2007) and worms (Tsytsugina & Polikarpov, 2003). The present study aims to address these data gaps by coupling laboratory based exposures of crustaceans with analysis of populations chronically exposed to radiation at Chernobyl.

1.4.5 Effects of Radiation on Mortality

Despite the recent trend towards studies using chronic exposures of sub-lethal doses, the available literature of radiation effects in crustaceans remains dominated by mortality studies. This data is often used to calculate lethal dose (LD_{50}) values (Dallas et al., 2012) in order to derive hierarchies of radiosensitivity across taxonomic groups (Blaylock et al.,1996; Harrison & Anderson, 1996). LD_{50} values are traditionally used in ecotoxicological studies to determine the ecological risk to species (Stark et al., 2004) and have also been employed in order to determine no observed effect concentration (NOEC) values (Garnier-Laplace et al., 2006).

Within the crustacean subphylum, the dose at which mortality occurs displays high variability (Dallas et al., 2012). Recent work using the harpacticoid copepod *Tigriopus japonicus* demonstrated tolerance of external gamma radiation doses of up to 600 Gy, with mortality only occurring 5 days after cessation of exposure (Han et al., 2014a). This is within the same order of magnitude as some bacteria and protozoan species, groups considered amongst the most radioresistant organisms (Copplestone et al., 2001). Furthermore, upon irradiation of dry egg masses, Iwasaki et al., (1971) demonstrated extreme radioresistance in *Artemia salina* nauplii of up to 2780 Gy one day after hatching. However, *Artemia* cysts display remarkable resistance to a range of stressors (MacRae, 2003) attributed to their greatly reduced metabolic and developmental activity prior to hatching and therefore are not considered representative of other crustacean species. Conversely, Rees (1962) reported a 30 day LD_{50} of ~15 Gy in the grass shrimp, *Palaemonetes pugio* which is within the upper bounds of radiosensitivity of some mammalian species (Blaylock et al., 1996).

Comparatively, the effects of chronic radiation doses on mortality in crustaceans have been underrepresented. Marshall (1966) exposed 25 populations of *Daphnia pulex* to external gamma radiation over a 55 week period for 18.5 hours a day with doses ranging from 0 to ~ 5.1 Gy/d. At the three highest dose levels (~5.1 Gy/d, ~4.8 Gy/d and ~ 4.36 Gy/d) populations crashed and became extinct, which the author attributed to an increase in individual death rate approaching the upper limit of the sustainable birth rate. Other monitored parameters such as the % of aborted eggs and embryos were shown to increase at dose levels below those leading to extinction, reiterating the greater sensitivity of reproductive endpoints comparable to mortality. Parisot et al., (2015) corroborated these findings, reporting a slight but non-statistically significant increase in mortality in ^{137}Cs

exposed *D. magna* at a dose rate of 35.4 mGy/hr, with sub-lethal impacts occurring at much lower dose rates of 0.007 mGy/hr.

Engel (1967) exposed blue crabs, *Callinectes sapidus*, to acute doses (maxium of 180 minutes exposure) of gamma radiation from ^{60}Co over a total dose range of 40 – 640 Gy at a dose rate of 219 Gy/hr. The author also continually exposed *C. sapidus* to dose rates of 0.032, 0.073 and 0.29 Gy/hr over a 70 day period. Following acute exposure a 30-day LD_{50} of 510 Gy was recorded. In contrast, crabs subjected to a total accumulated dose of ~ 460 Gy at the dose rate of 0.29 Gy/hr over a 70 day period displayed 100% mortality.

1.4.6 Summary

Despite numerous international directives and decades of research into the biological effects of radiation, significant knowledge gaps remain. For example, the majority of available data is derived from acute studies which are not environmentally relevant. Within the FREDERICA database, 64% of the data points were obtained following acute radiation exposures, with 36% following chronic exposures. Furthermore, the available chronic data are heavily biased towards fish, mammals and terrestrial plants with a scarcity of data evident for crustaceans. Another major limitation is the discrepancy between the available data for laboratory toxicity tests comparable with field studies. The majority of field data is heavily biased towards small mammals; (Baker et al., 1996; Beresford et al., 2008; Chesser et al., 2000), fish (Dallas et al., 1998; Jonsson et al., 1999; Sugg et al., 1996), plants (Kovalchuk et al., 1998, 2000; Syomov et al.,1992) and birds (Bonisoli-Alquati et al., 2010; Galván et al., 2014; Hermosell et al., 2013). Comparatively, the majority of field studies regarding crustacean communities exposed to radionuclides are focused on bioaccumulation of radioactive materials (Marzano et al., 2000) relating to trophic transfer, or calculating estimates of received doses (Murphy et al., 2011; Vives i Batlle et al., 2014). Radiation effects have been shown to occur at different dose rates between controlled and field exposures. Garnier-Laplace et al., (2013) analysed available radioecology data of terrestrial wildlife and found effects occurred at dose rates eight times lower in the field as compared to controlled exposures, though this was based on only limited field data. This underpins the need for combined laboratory and field studies of biota to understand the applicability of controlled exposures to natural populations. Finally, this review has highlighted the lack of environmentally relevant data regarding the effects of radiation on crustaceans across a range of commonly used endpoints.

1.5 Project Aims

Considering the data gaps identified in the preceding review and Fuller et al., (2015), the aims of this PhD project were;

- To elucidate the biological effects of chronic, environmentally relevant doses of ionising radiation on crustaceans across a range of phenotypic and genetic endpoints
- To determine whether chronic exposure to low levels of radiation in the environment is significantly impacting crustaceans at contaminated sites
- To establish the applicability and relevance of laboratory radioecology studies with respect to the natural environment
- To assess the efficacy of current proposed benchmarks for protection of the environment from ionising radiation

Chapter 2

Effects of Ionising Radiation on Male Fertility, Reproduction and DNA Damage in Marine and Freshwater Amphipods

2.1 Introduction

2.1.1 Effects of Radiation on Male Fertility

Elucidating the effects of environmental contaminants on male fertility has become a priority issue over the last twenty years owing to a sharp increase in the incidence of male reproductive disorders and declining global sperm counts (Sharpe & Skakkebaek, 1993; Toppari et al., 1996). Consequently, the use of vertebrate fertility assays in ecotoxicology and knowledge of male reproductive systems has increased (Kumar et al., 2013; Volkova et al., 2015; Wisniewski et al., 2015). Ionising radiation has been shown to have deleterious impacts on male fertility in a range of organisms including fish (Knowles,1999), birds (Møller et al., 2014) and rodents (Gong et al., 2014). Ionising radiation affects molecules both by direct damage and indirectly via generation of reactive oxygen species (ROS) that may cause oxidative stress. Sperm cells are thought to be susceptible to damage from ROS owing to limited anti-oxidant machinery, high metabolic activity and the high polyunsaturated fatty acid content of their membranes (Aitken et al., 2016; Sikka, 2001; Tremellen, 2008). Oxidative stress (defined here as an imbalance between antioxidants and oxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage, Sies & Jones, 2007) has been shown to be detrimental to a range of sperm quality parameters including motility, morphology and fertilization potential (Tremellen, 2008). Furthermore, direct and indirect induction of DNA damage by IR (See Sect 2.1.2) may persist in sperm cells owing to down-regulation of DNA repair and loss of apoptotic capabilities during spermatogenesis (Lewis & Aitken, 2005; Marchetti & Wyrobek, 2008). This may lead to important transgenerational effects, including developmental abnormalities in the resultant embryos (Aitken & De Iuliis, 2007)

Following the Chernobyl accident, a number of studies reported an increase in morphological abnormalities and perturbations to spermatogenesis in clean-up workers exposed to high levels of radiation (Bartoov et al., 1997; Cheburakov & Cheburakova, 1992; Fischbein et al.,1997). Deleterious effects have also been recorded in non-human biota inhabiting highly contaminated areas within the Chernobyl exclusion zone. Bonisoli-Alquati et al., (2011) studied the relationship between sperm motility in barn swallows, *Hirundo rustica*, at Chernobyl and plasma oxidative status to determine the importance of individual anti-oxidant status in observed effects on sperm. The authors found deleterious effects of radiation exposure on sperm motility that were dependent upon individual antioxidant status, though not all sperm parameters were negatively associated with radiation. However, in a modelling study of the impact of Chernobyl relevant dose rates on oxidative stress, Smith et al., (2012) concluded that direct oxidative damage is unlikely to be the mechanism for observed effects on organisms. At the highest dose rates of radiation present at Chernobyl and Fukushima, models suggested no significant changes in antioxidant concentrations or cellular redox potential.

Recent studies have focussed on sperm parameters in organisms chronically exposed to low-dose radiation at Fukushima. For example, Okano et al., (2016) monitored effects of chronic low-dose exposure on sperm cell apoptosis and sperm morphological parameters in the Japanese field mouse, *Apodemus speciosus*. The authors found no increase in the frequency of apoptotic cells or morphologically abnormal sperm cells in *A. speciosus* at sites highly contaminated by the Fukushima accident (ambient dose rates up to 13.9 µSv/hr). Similarly, no impacts of radiocaesium on sperm morphology and spermatogenesis was observed in Japanese black bull (Yamashiro et al., 2013) following exposure to mean total doses (external and internal) of 8 mGy. The impacts of low-dose radiation on fertility are clearly not ubiquitous and necessitate further study in both the laboratory and the field.

Despite the renewed interest in the impacts of environmental contaminants on male fertility, effects on aquatic invertebrates remain poorly understood (Lewis & Ford, 2012). Studies are heavily biased towards female reproductive success, with typical endpoints being number of eggs produced, hatching success or timing of reproduction. A small number of studies have demonstrated the use of aquatic invertebrate infertility assays in assessing pollutant impacts. For example, Yang et al., (2008) studied the effects of industrial pollution on sperm numbers in the marine amphipod crustacean, *Echinogammarus marinus*, at sites of varying contamination in Scotland, UK. A 20% reduction in the numbers of sperm in individuals inhabiting contaminated sites was recorded as compared to less polluted sites. To the author's knowledge, no study has documented radiation-induced effects on male fertility in an aquatic invertebrate. The present study aimed to address this knowledge gap by providing a robust understanding of the effects of low-dose IR on fertility in two amphipod crustacean species.

2.1.2 Genotoxic Effects of Radiation

DNA damage drives a number of biological processes including mutagenesis, carcinogenesis and ageing (De Bont & van Larebeke, 2004). Energy deposition by ionising radiation generates a wide range of damage to DNA molecules, including single and double strand breaks (DSBs). Damage is caused by both direct ionisation and indirectly via generation of hydroxyl radicals that attack DNA (Hada & Georgakilas, 2008). Oxidative DNA damage occurs naturally from processes such as aerobic metabolism, with as many as 50000 lesions daily per cell (Swenberg et al., 2011). However, DNA damage induced by IR is of greater significance due to the complex clustering of damage and presence of double strand breaks, which are rarely produced due to endogenous processes (see Lomax et al., 2013 for review). Structurally and chemically complex clustered DNA damage sites are a typical signature of IR-induced damage, and are thought to have reduced reparability as compared to more homogenous DNA damage from endogenous processes (Eccles et al., 2011). Unrepaired DNA damage may lead to deleterious impacts such as oncogenesis, mutagenesis, cell death and developmental abnormalities (Hanawalt, 2002). Consequently, the assessment of radiation-induced DNA damage is fundamental in oncology, human biology and ecotoxicology. Within ecotoxicology studies, genotoxicity analyses are often favoured as they are considered more sensitive than conventional endpoints such as mortality or reproduction (Jha, 2008 but see Forbes et al., 2006).

A number of studies have focused on the genotoxic effects of environmental radiation on aquatic organisms. Sugg et al., (1996) studied the effects of Chernobyl-derived radiation on DNA damage in the channel catfish, *Ictalurus punctatus*, using the alkaline unwinding assay and the micronucleus test. The authors recorded a significant relationship between radiocaesium content of muscle tissue and genetic damage as measured using the alkaline unwinding assay, though incidence of micronuclei was not significantly elevated. Similarly, AlAmri et al., (2012) recorded an increase in DNA damage and gene expression of RAD51, a key gene involved in the repair of DSBs, in the blue mussel *Mytilus edulis* at a site contaminated with radionuclides as compared to a control site. Calculated dose rates of 0.61 µGy/hr to *M. edulis* were found to be sufficient to induce a genotoxic response, highlighting the sensitivity of genotoxicity analyses.

As highlighted in Sect 2.1.1, DNA damage is of particular importance in sperm cells due to the potential for transmission across generations and subsequent effects on progeny. In humans, occupational exposure to ionising radiation has been shown to increase DNA fragmentation in sperm cells as measured by the comet assay (Kumar et al., 2013). The comet assay, or single cell gel electrophoresis, is the most commonly used method for assessing genotoxicity, and provides a simple robust measure of DNA damage within individual eukaryotic cells (Kumaravel & Jha, 2006; Olive & Banáth, 2006). Single cells are embedded in agarose on a slide and following removal of cellular proteins by lysis, subject to electrophoresis under alkaline conditions (Singh et al., 1988). Damaged DNA migrates towards the anode owing to relaxation of supercoils, forming a ‘comet-like’ structure (Fairbairn et al., 1995). The amount of migrated DNA in the ‘tail’ of the comet is proportional to the amount of DNA damage.

In non-human biota, a range of studies have demonstrated induction of DNA damage in sperm following exposure to model genotoxicants. For example, Lewis & Galloway (2009) recorded elevated DNA damage in the sperm of *Mytilus edulis* and *Arenicola marina* exposed to benzo(a)pyrene and methyl methanesulfonate (MMS), which in turn lead to an increase in offspring displaying developmental abnormalities. Similar results have been demonstrated in the freshwater amphipod, *Gammarus fossarum*, and the three-spined stickleback (*Gasterosteus aculeatus*) exposed to MMS (Devaux et al., 2011; Lacaze et al., 2011). At present there is a significant data gap regarding the effects of IR on DNA integrity in the sperm of non-mammalian organisms. To the author's knowledge, no study has focussed on the effects of environmentally relevant doses of IR on sperm DNA damage in an aquatic invertebrate.

2.1.3 Amphipods as Model Organisms in Environmental Monitoring

Amphipoda (Crustacea) are one of the major benthic components of freshwater and marine systems worldwide, both in terms of biomass and species diversity (Thomas, 1993). Amphipods are ecologically important in the transfer of nutrients to higher trophic levels such as fish or seabirds, and are often intermediate hosts of parasites. Owing to their global abundance, ecological importance and sensitivity to a range of pollutants, amphipods are commonly used as bioindicators (Hart & Fuller, 1979). For example, Gesteira & Dauvin (2000) demonstrated the efficacy and greater sensitivity of amphipod species as bioindicators compared to other macrobenthic species following the *Amoco Cadiz* and *Aegean Sea* oil spills.

Amongst the most commonly used amphipods in environmental studies are members of the family Gammaridae. Gammarid amphipods are frequently used to monitor the impacts of anthropogenic contaminants, and have been studied in response to a range of xenobiotics including pharmaceuticals (De Lange et al., 2009; Guler & Ford 2010), pesticides (Agatz et al., 2014; Zubrod et al., 2015) and metals (Prygiel et al., 2016; Ternjej et al., 2014). In recent years, gammarid amphipods have been commonly used to assess the genotoxic effects of a range of environmental pollutants (Lacaze et al., 2010; Neuparth et al., 2005; Ternjej et al., 2014). To the authors knowledge however, only two studies have focussed on the effects of IR on gammarid amphipods. Hoppenheit (1973, 1980) exposed the brackish water amphipod, *Gammarus duebenii*, to both X-rays and the transuranium radionuclide, Americium-241 at acute and chronic doses of up to 6.5 Gy and 30.2 mGy/hr respectively. X-ray exposure caused a reduction in the fecundity of female *G. duebenii* at doses of 3 Gy, whilst survival and moulting rate were unaffected. Chronic exposure to ^{241}Am caused elevated mortality at dose rates of 30.2 and 3.4 mGy/hr, whilst moulting was unaffected. The dose rates used in the previous studies are orders of magnitude above typical environmental exposures. In addition, organisms are not typically exposed to x-rays in the natural environment, limiting the environmental relevance of the former study. The present study aims to address this data gap by elucidating the effects of chronic, environmentally relevant doses of radiation on two ecologically important gammarid species, *Echinogammarus marinus* and *Gammarus pulex*.

i) Echinogammarus marinus

Echinogammarus marinus (Crustacea:Amphipoda) is a widespread intertidal amphipod with a distribution ranging from Norway to Southern Portugal (Maranhão et al., 2001; Alexander et al., 2013). *E. marinus* is omnivorous, both grazing on algal species and actively predating on a range of macroinvertebrates (Dick et al., 2005). *E. marinus* has gained prominence as a model organism in a range of disciplines including ecology (Guler et al., 2012; Maranhão et al., 2001), reproductive biology (Ford et al., 2003; Maranhão & Marques, 2003) and ecotoxicology (Bossus et al., 2014; Guler & Ford, 2010; Yang et al., 2008). *E. marinus* reproduces continuously throughout the year and is highly abundant, allowing for no limitations on conducting reproductive studies and emphasising its potential as a model organism.

ii) Gammarus pulex

Gammarus pulex is an important species in freshwater systems, being the most abundant native amphipod species in many areas of Western Europe (Maazouzi et al., 2011). *G. pulex* plays a key ecosystem role in leaf litter degradation and thereby nutrient cycling (Maltby, 2002). Owing to its ecological importance, *G. pulex* has been used extensively in ecotoxicological research with an emphasis on feeding assays and measures of leaf shredding efficacy (Åbjörnsson et al., 2000; Maltby et al., 2002; Zubrod et al., 2010).

2.1.4. Reproduction in Amphipods

Amphipod crustaceans exhibit precopulatory mate guarding behaviour, wherein the male guards the female for up to a two week period prior to mating (Elwood & Dick, 1990). In females, the ovarian cycle is closely synchronised with moulting events. Consequently, females are only available for mating during a brief period, necessitating mate guarding behaviours (Hyne, 2011). Comparatively, male amphipods can mate throughout the majority of the moult cycle once sexually mature (Sutcliffe, 1992). Male amphipods allocate substantial numbers of sperm to each mating event, though Lemaître et al., (2009) found that this did not impair subsequent male reproduction in *G. pulex*. In *G. pulex* and the brackish water amphipod, *Gammarus duebenii*, sperm numbers are estimated at approximately 10,000 per testis (Lemaître et al., 2009). Larger numbers of ~ 60,000 and a positive correlation between individual body weight and sperm numbers in *E. marinus* were recorded by Yang et al., (2008). The precopulatory mate guarding (amplexus) phase ends once the female moults and external fertilization occurs wherein the male deposits sperm within the brood pouch (Hynes, 1955). Embryonic development occurs within the brood pouch for a duration of roughly 20 days. During this time, progeny undergo six developmental stages and emerge as fully grown young (McCahon & Pascoe, 1988).

2.1.5 Selection of Exposure Conditions

Dose rates of 0, 0.1, 1 and 10 mGy/d were selected based on typical environmental exposures and proposed regulatory guidelines for the protection of individuals (see Section 1.1.3). These dose rates allowed testing of the suitability of regulatory limits proposed for the protection of aquatic populations, and permit prediction of the consequences of radioactive releases to the environment. In the initial period following the Fukushima accident for example, dose rates of 1 mGy/d to crustaceans were calculated by the IAEA

(2015). For the ICRPs reference crustacean, deleterious effects are anticipated over a band of dose rates from 10 – 100 mGy/d. The results of the present study will therefore aid in the derivation of robust environmental radioprotection dose limits.

The beta emitter phosphorus-32 (^{32}P) was used as the exposure source in the present study. Phosphorus-32 is used as a proxy for the beta/gamma doses that are primarily received by human and non-human organisms following nuclear incidents (Petersen, 1965; Priyadarshi et al., 2011; Straume et al., 2003). In addition, ^{32}P is released to the environment from medical and research establishments owing to its uses in radiotherapy and as a tracer in genetic studies (Smith et al., 2011), meaning organisms may be exposed to ^{32}P in the natural environment. The characteristics of ^{32}P are shown in Table 2.1. The effects of ^{32}P on crustaceans have not been studied. Within the ERICA tool (v 1.2), a modelling software system implemented to assess radiological risk to non-human biota (Brown et al., 2008), no data exists for the concentration ratio (CR, defined in Formula 2.1) of ^{32}P in crustaceans. Derivation of accurate CR values is fundamental in providing robust estimates of dose rates received by biota (Wood et al., 2013). This necessitated a preliminary study into the uptake of ^{32}P by model amphipod species in order to ensure accurate dose rates were achieved.

$$CR = \frac{R_{act}\ \ in\ biota\ whole\ body\ fresh\ mass\ (Bq\ kg^{-1})}{R_{act}\ in\ water\ (Bq\ l^{-1})}$$

Formula 2.1 – Concentration ratio of a given radionuclide in aquatic organisms. From (Hosseini et al., 2008)

Table 2.1 – Characteristics of phosphorus-32. Data from Delacroix et al., (2002) .

Phosphorus-32	
Half life	14.3 Days
Decay Product	Sulphur-32
Decay Mode	Beta (100%)
Decay Energy	1.71 MeV

2.1.6 Objectives & Hypotheses

Given the data gaps identified in the above section, the present study aimed to:

a) Determine and compare the uptake of ^{32}P by the model amphipod species, *Echinogammarus marinus* and *Gammarus pulex* to derive novel CR values

b) Elucidate the effects of ionising radiation on male fertility in *Echinogammarus marinus* and *Gammarus pulex* and potential knock-on effects on reproduction

c) Assess the impact of environmentally relevant doses of radiation on DNA damage in sperm cells of amphipod crustaceans

Owing to the known detrimental impact of IR on male fertility and previous studies demonstrating pollutant impacts on sperm in amphipods (e.g. Yang et al., 2008), it was hypothesised that:

- IR would cause a reduction in both the number and quality of sperm produced by both *E. marinus* and *G. pulex*.
- Perturbations to sperm would be linked with an increase in DNA damage, and concomitant effects on female reproduction such as a reduction in fecundity and increased developmental abnormalities in progeny.

2.2 Materials & Methods

2.2.1 Collection & Culturing of Echinogammarus marinus and Gammarus pulex

i) Echinogammarus marinus

E. marinus were collected from Lock Lake in Langstone Harbour, England, UK (50°47′23.13N, 1°02′37.25W), an area designated as a special protection area (SPA), a site of special scientific interest (SSSI) and an internationally important region for wading birds (Bossus et al., 2014). Individuals were collected by hand from underneath fucoid seaweed and stones at low tide periodically from September 2015 to October 2016. Collected *E. marinus* were housed at the Institute of Marine Sciences (IMS, Portsmouth, UK) in aquaria containing aerated seawater at 10°C with a photoperiod of 12 hours light 12 hours dark. Individuals were fed *ad libitum* on mixed brown fucoid seaweed. Prior to experimentation, animals were sexed by analysis of the uropods following the description of Sexton & Spooner (1940) and visually inspected for parasitic (trematode) infection. Adult males were then acclimated to aerated artificial seawater, AFSW, (33ppt, Peacock Salt, composition available at https://www.peacocksalt.com) for at least two weeks prior to experimentation.

ii) *Gammarus pulex*

G. pulex were collected using a 500 µm mesh hand net from the River Ems, England, UK, (N50°51'34.8",W0°55'45.8") periodically from April 2015 to February 2016. Historic data from the UK Environment Agency designates this stretch of the river as a 'grade A', specifying 'very good' water quality (De Castro-Català et al., 2017, http://apps. environment-agency.gov.uk). Upon return to the laboratory, individuals were sorted out from other macroinvertebrates and maintained in river water collected from source at time of sampling. *G. pulex* cultures were kept at 15 °C with a twelve hour light dark cycle. Prior to experimentation, individuals were sexed by analysis of the posterior gnathopods (Hume et al., 2005) and visually inspected for presence of acanthocephalan parasites. Adult males with no signs of infection were kept in aerated mineral water (Artificial Freshwater, AFW) (Volvic, France) and fed Elm leaves (*Ulmus x hollandica* 'Vegeta') collected from Southsea

Common (50°46'47.8"N, 1°05'15.4"W) for two weeks prior to experimentation. Leaves were pre-conditioned according to the methods of Bloor (2010) whereby organic detritus from the sampling site is mixed with leaves for at least ten days to allow for fungal colonisation.

2.2.2 Experimental Design for P-32 Exposures

All individuals were exposed to solutions of ATP γ-^{32}P (Perkin Elmer UK, NEG002A100UC) and AFSW/AFW for *E. marinus* and *G. pulex* respectively. For initial uptake experiments, a total of 20 male adult *E. marinus* and *G.* pulex were exposed to solutions with activity concentrations 9.6 ± 0.85 Bq/ml and 5.71 ± 0.72 Bq/ml respectively. Based on CR values from uptake experiments and calculations using the ERICA tool (v 1.2), solutions of 62.9 Bq/ml and 14.8 Bq/ml were prepared for 10 mGy/d exposures of *E. marinus* and *G. pulex* respectively. Dilutions of 10 and 100-fold were used for 1 and 0.1 mGy/d treatments. Control solutions were AFW and AFSW for *E. marinus* and *G.* pulex. Structurally analogous non-radioactive phosphorus (Adenosine Triphosphate [ATP], New England Biolabs, UK) of the same molar concentration was added to all treatments excluding the highest dose rate to ensure total phosphorus remained constant amongst all treatments. A total of 24 individuals were used per treatment for all experiments excluding preliminary uptake exposures. All experiments were conducted for 14 days.

For all exposures, individuals were housed in 50 ml glass beakers (Fisher Brand, Squat Form) containing 20 ml of exposure solution. This volume was chosen in compliance with University regulations regarding the permissible discharge of radioactive materials. Glass beakers were used preferentially owing to preliminary observations of ^{32}P adherence to plastic containers. *E. marinus* individuals were kept at 10°C with a 12 hour light 12 hour dark cycle fed on 100 mg pieces (± 10%) of brown fucoid seaweed. *G. pulex* were kept at 15 °C under the same photoperiod and fed on 50 mg (± 10%) pre-conditioned elm leaves. In both species, water changes of 18 of the 20ml of exposure solution were conducted every three days. Individuals were housed within a Panasonic MIR-254 cooled incubator for all exposures. Ambient dose rates within the incubator were measured using a Mini Monitor

900 to give an indication of background radiations. Dose rates were not found to exceed 0.10 µGy/hr, indicating no elevation above background levels. Both species were monitored daily for moults or deaths. Individuals of both species were removed and analysed for ^{32}P activity concentrations at every water change along with the corresponding exposure solution. For the preliminary uptake experiment, activity concentrations were measured in whole samples, dissected hepatopancreas tissue and exoskeleton.

All activity concentration measurements were conducted using a HIDEX 300SL liquid scintillation counter and associated MikroWin 2000 software (Version 4.43). For aqueous samples, 1 ml of exposure solution was added to a ViaLogic (Lab Logic, UK) plastic scintillation vial containing 9 ml of Scint Logic U (Lab Logic, UK) scintillation cocktail and mixed rigorously. All solid samples were first weighed using a five-point sensitive balance (Sartorius R180D, Sartorius, Germany) and homogenised using a micropestle before addition to a scintillation vial containing 9 ml cocktail. Samples were mixed thoroughly and dark acclimated for one hour at room temperature prior to counting. All measurements were conducted in triplicate with a 10 second count time and 3 range repeats per sample. Adequate counting efficiency was validated by triple-to-double coincidence ratio (TDCR) values of > 0.9, suggesting a very high accuracy of counts (Broda, 2003).

2.2.3 Derivation of Concentration Ratios and Dose Calculations using the ERICA Tool

Ten adult male *E. marinus* and *G. pulex* were geometrically measured to generate accurate reference organisms for dose assessment using the ERICA tool (see Table 2.2). Calculated dose conversion coefficients (DCCs) were 2.52 E-04 and 1.49 E-04 µGy/hr per Bq/kg for internal and external beta-gamma radiation respectively in *E. marinus*. In *G. pulex*, DCCs of 2.35 E-04 and 1.64 E-04 µGy/hr per Bq/kg for internal and external beta-gamma respectively were calculated. Concentration ratios were derived following Formula 2.1 (Hosseini et al., 2008).

Table 2.2. – Geometric parameters used for creation of the *E. marinus* and *G. pulex* reference organisms in the ERICA Tool.

Parameter	***Echinogammarus marinus***	***Gammarus pulex***
Length (mm)	20.74	9.21
Width (mm)	1.92	1.30
Height (mm)	3.99	1.84
Mass (g)	0.104	0.038

2.2.4 Male Fertility Assays in E. marinus and G. pulex

I) Reagents Used

Sperm Buffer

- 20 ml Leibovitz L-15 Medium, without phenol red (Sigma-Aldrich, UK)
- 47.6 mg HEPES (Sigma-Aldrich, UK)

Add HEPES to pre-chilled L-15 medium and vortex for 10 seconds. Store at 4 °C.

Working Anaesthetic Solution

- 3.7 ml Clove Oil (Numark, UK)
- 16.3 ml 96% Ethanol (Sigma-Aldrich, UK)
- 25 ml AFSW
- 25 ml AFW

Add clove oil to 96% ethanol and mix thoroughly for stock anaesthetic solution. Add 80 µl of stock solution to AFSW or AFW dependent on test organism.

SYBR-14 Live Sperm Stain, 10 µM

- 5 µl 1mM SYBR-14® (Molecular Probes)
- 4995 µl Dimethyl Sulfoxide (DMSO, Thermo Scientific)

Add 1mM SYBR-14® to Dimethyl Sulfoxide and vortex for 10 seconds. Store in darkness at 4°C.

Dead Cell Stain

- 2.4 mM Propidium Iodide (PI, Molecular Probes)

Store at –20 °C

II) Protocol

Following exposure to IR, individuals were anaesthetised using the working anaesthetic solution described above and weighed using a four-point balance with a precision of ± 0.1 mg (Fisher Brand, MH-124). Removal of the head, hepatopancreas, first pereon segment and the posterior section from the second pleosome was first performed to avoid accidental damage to the testis (see Figure 2.1). Using a pair of fine dissecting scissors (5mm blade length), the cuticle was cut along the ventral midline lengthways in order for testes to be removed (Ford et al., 2005). Dissected testes were placed in 20 µL of sperm buffer on a cavity slide and dilacerated to obtain a sperm solution. A range of different buffers were tested in a pilot experiment to ascertain the optimum conditions for maintaining viability of dissected testes and sperm cells.

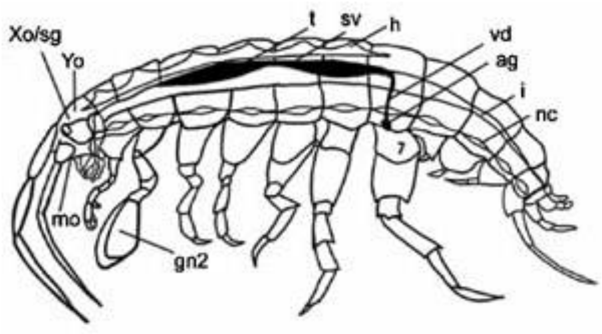


Figure 2.1 - Lateral view of a typical male gammaridean amphipod with reproductive system indicated in relation to other visceral structures. From Hyne (2011). t = testes, sv = seminal vesicle, vd = vas deferens, ag = androgenic gland, h = heart, gn2 = second gnathopod, I = intestine, nc =nerve cord, mo = mandibular organ, Xo/sg = X/organ sinus complex.

The sperm solution was transferred by micropipette to a pre-weighed 1.5 ml microcentrifuge tube containing 20 µL of pre-chilled sperm buffer. The solutions were thoroughly mixed by pipetting and vortexed for 10 seconds. Sperm solutions were reweighed to obtain the exact volume of liquid and kept on ice. Sperm viability was assessed using a LIVE/DEAD viability kit (Molecular Probes Inc, L-7011,). This kit contains two fluorescent dyes: SYBR-14®, a membrane permeant nucleic acid stain, and propidium iodide (PI), a membrane-impermeable conventional dead cell stain (Nagy et al., 2003). Both of the dyes label DNA, offering an advantage over conventional cell stains that target different cellular components which may confound viability analyses. LIVE/DEAD analyses have been shown to provide robust measures of sperm viability in a range of organisms including bulls (Nosrati et al., 2014), goats (Turri et al., 2014) and humans (Amaral et al., 2011). 5 µL of 10 µM SYBR-14 solution was added to sperm solutions and vortexed for 10 seconds. Following dark incubation at room temperature for 5 minutes, 5 µL of 2.4 mM PI was added, mixed thoroughly and vortexed for another 10 seconds. Following dark incubation for a further 5 minutes, 5 µl aliquots of stained sperm solutions were added to the upper and lower grids of a Neubauer haemocytometer and a 22 x 22 mm cover slip (BDH, Kuwait) added. Slides were visualised using a Leica DM2000 fluorescent microscope with filters of 340 – 480 nm and 450 – 490 nm for SYBR-14 and PI staining respectively. An example of live/dead stained *E. marinus* spermatozoa is shown in Figure 2.2. Numbers of live and dead sperm were counted in three randomly selected grid squares of the haemocytometer and three replicate counts performed. Sperm viability (% of live sperm) and total sperm count in the solution was calculated. Analysis of male fertility was conducted on a total of 148 male *E. marinus* over two separate exposures. For *G. pulex*, a total of 61 individuals were analysed following a single exposure experiment.

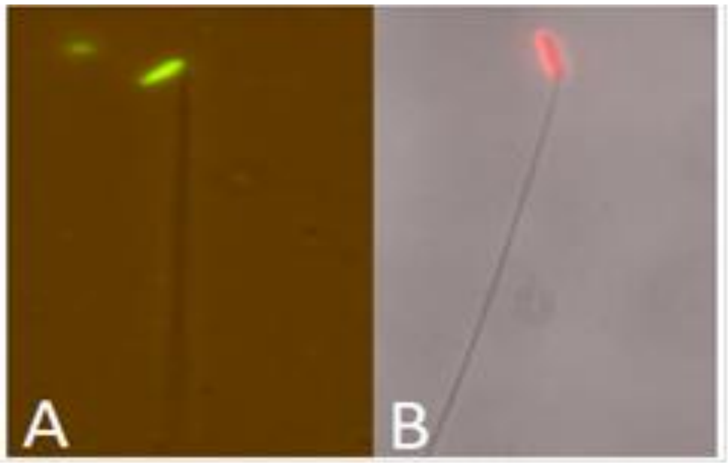


Figure 2.2 – Example of an A) live and B) dead *Echinogammarus marinus* spermatozoa stained with SYBR-14 and propidium iodide respectively.

2.2.5 Breeding Experiment

To determine potential knock-on effects of perturbations to sperm quality and quantity, males exposed to IR were allowed to reproduce with non-exposed females. Following exposures to 0, 0.1, 1 and 10 mGy/d (see Section 2.2.2 for exposure design) for two weeks, a total of 81 male *E.marinus* were paired with a sexually mature female. Sexually mature females were defined as those in amplexus (Watts et al., 2002). Females were first separated from pairs in culture following the method of Malbouisson et al., (1995), whereby paired individuals are placed on filter paper until the male lets go of the female. Sexually mature females were acclimated to AFSW for two weeks under the culture conditions described in section 2.2.1. Females were then weighed and added to 100 ml plastic containers containing 50 ml of AFSW. Exposed males were first weighed and added sequentially to the containers containing mature females. In gammaridean amphipods, size assortative mating has been observed whereby large males preferentially pair with larger females (Elwood et al., 1987) necessitating accurate records of the weight of the pair. Brown fucoid seaweed was added for feed and the time of male addition recorded.

Observations of pairing status (paired or unpaired), moulting and mortality were observed daily until all pairs had formed. Water changes with fresh AFSW were conducted every three days and feed replenished. In gammarids, copulation occurs following moulting of the female in amplexus (Plaistow et al., 2003). Once females had moulted and were ovigerous (bearing eggs), males were immediately removed from test containers and analysed for sperm quality and quantity following the protocol in section 2.2.4. After five days post-reproduction, ovigerous females were reweighed, anaesthetised and embryos were removed from the marsupium using a plastic Pasteur pipette and forceps. Embryos were visualised and photographed using a Leica MZ10F dissecting microscope with mounted Leica DFC130 camera. Photographs were randomly coded by a colleague to ensure all subsequent analysis was conducted blind. The number of eggs per individual and % embryo abnormalities per female were calculated following Sundelin & Eriksson (1998) and Sundelin et al., (2008).

Abnormalities were scored as abnormal or normal, with no distinction of the severity of the aberration. Examples of typical abnormalities are shown in Figure 2.3. Embryo diameters were measured using Image J (v1.48).

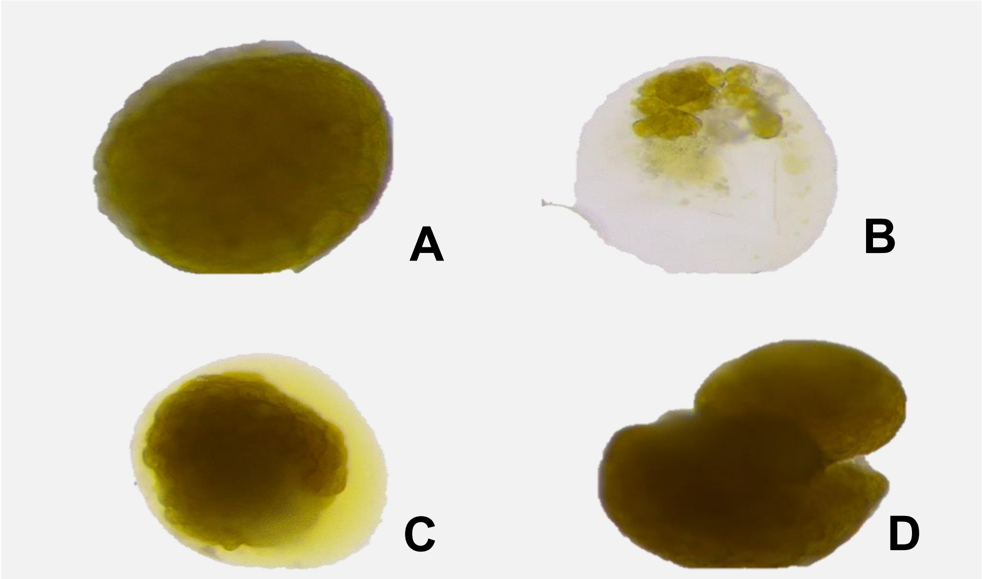


Figure 2.3. – Embryo abnormalities in *E. marinus.* A = Typical healthy *E. marinus* embryo. B = Unfertilised embryo. C = Embryo with impaired membrane and lipids leaking in to egg sac. D = Malformed embryo.

2.2.6 Single Cell Gel Electrophoresis in E. marinus *Sperm Cells*

I) Reagents Used

Alkaline Electrophoresis Solution

- 12g NaOH Pellets (Fisher Scientific, UK)
- 2 ml 500 mM EDTA, pH 8.0 (Sigma-Aldrich, UK)

Make up to 1L with distilled water. Final pH of 13.6 measured with Mettler Toledo pH meter and electrode (LE409). Stored at 4°C

Neutralisation Buffer

- 12.6 g Tris-Hydrochloride (Fisher Scientific, UK)

Make up to 200 ml with distilled water. pH buffered to 7.5 using NaOH. Stored at 4°C.

Lysis Buffer (TREVIGEN, Gaithersburg, MD, USA)

Stored at Room Temperature.

1 x TAE Buffer (National Diagnostics, UK)

Stored at 4°C

1 x SYBR Gold in 1 x TAE

- 10,000 x SYBR Gold in DMSO (Molecular Probes)
- 1 x TAE

Add 10 µl 10,000 X SYBR Gold to 100ml of 1 x TAE. Store at – 20°C.

1% Agarose

- 0.25 g Low Melting Point Agarose, (Thermo Scientific, UK)

Make up to 25 ml with distilled water.

70% Ethanol (VWR, UK)

Stored at room temperature.

II) Protocol

The protocol for single cell gel electrophoresis in *E. marinus* sperm cells was adapted from Lacaze et al., (2011) with some modifications. Viability and number of sperm cells was first assessed using the methods described in Sect 2.2.4. Any sperm suspensions with a cell density of greater than 180 cells per µL were diluted two-fold with L15 medium prior to comet analysis. Cell density is an important consideration when performing the comet assay. Dense cell suspensions with overlapping comets creates problems in accurate scoring, whilst less dense suspensions may preclude an adequate number of comets for robust analysis (Collins, 2014). Sperm solutions were mixed with 1% agarose preheated to 37 °C at a ratio of 1:1 and 30 µl was spread on a 20-Well CometSlide HT® (TREVIGEN, Gaithersburg, MD, USA). This process was repeated for 20 sperm solutions per slide randomly between treatments. Slides were incubated in darkness at 4°C for 7 minutes for polymerisation of the agarose. Slides were then submerged in lysis solution and incubated in darkness for 18 hours. A preliminary experiment demonstrated that 18 hours was the optimum duration for sperm cell lysis, owing to greater chromatin condensation in sperm cells as compared to somatic cells wherein protocols adopt lysis times of 1 or 2 hours (Evenson et al., 2002; Lacaze et al., 2010). Following lysis, slides were washed twice for 5 minutes at room temperature with distilled water. Slides were immediately placed in an electrophoresis tank (GeneFlow, UK) containing approximately 600ml of freshly prepared alkaline electrophoresis solution. Electrophoresis was performed at 24V, 405 mAmp for 15 minutes. Slides were washed twice for 5 minutes with neutralisation buffer, followed by one 5 minute wash with 70% ethanol. Slides were then archived for later staining and comet scoring. All the steps described above were performed either in darkness or under yellow light to prevent induction of DNA damage from UV light (Lacaze et al., 2010).

Slides were stained by pipetting 60 µl of 1 x SYBR Gold on to each individual spot and incubation at room temperature for 5 minutes. Following a brief wash with distilled water to remove excess stain, slides were allowed to air dry in darkness. Slides were visualised using a Leica DM2000 high-powered fluorescence microscope and five random photographs taken per spot under 100 x magnification using a LEICA DFC130 camera. Comet images were randomly coded by a colleague to prevent bias and analysed using the OpenComet v1.3 software (Gyori et al., 2014). OpenComet is a free, open source software system that provides robust automated assessment of comet images that removes the subjectivity of visual analysis (see Figure 2.5, Końca et al., 2003). Percentage of tail DNA was used as a measure of DNA damage in the present study following the suggestions of Kumaravel & Jha (2006). A preliminary study using a 15 minute hydrogen peroxide exposure to induce sperm DNA damage (Hughes et al.,1996) was conducted to validate the efficacy of the comet protocol (see Figure 2.4).

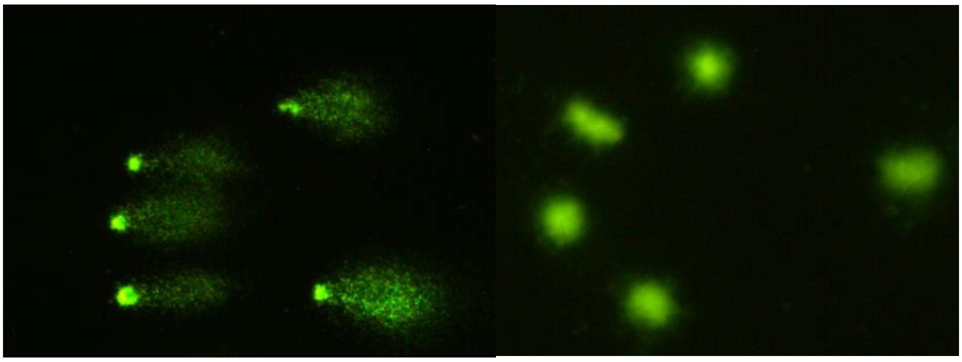


Figure 2.4 - Control (right) and 500 µM H_2O_2 exposed (left) *E. marinus* sperm cells stained with SYBR-Gold.

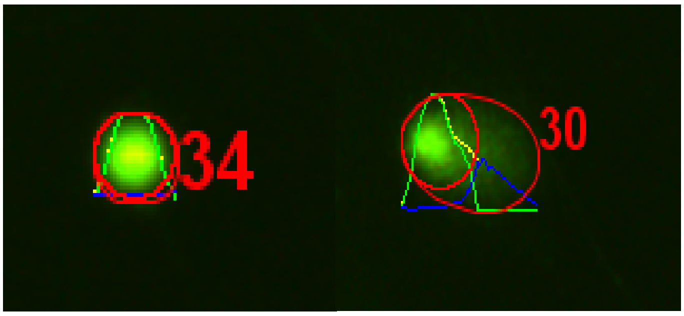


Figure 2.5 – Example of Open Comet Analysis measuring % Tail DNA in an undamaged *Echinogammarus marinus* sperm cell (left) and a sperm cell exhibiting DNA damage (right).

2.2.7 Statistical Analyses

i) Uptake and Moult/Mortality Data

Pooled moult data for all experiments was analysed using a generalized linear model (GZLM) using the R package lme4 (Bates et al., 2014). Generalized linear models allow for response variables with non-normal distributions, and were used to account for the binomial nature of the moult dataset (e.g. 1 = individual moulted, 0 = individual did not moult). Time (day of experiment), dose rate and trial were used as predictors for binomial moult count data. Differences between species in CR values were analysed using a Kruskal-Wallis H Test owing to the non-normal distribution of the dataset. To assess differences in ^{32}P accumulation between whole individuals, exoskeleton and hepatopancreatic tissue, a repeated measures analysis of variance (ANOVA) design was used to account for multiple measurements conducted on the same individual.

ii) Sperm and DNA Damage Analyses

An analysis of covariance (ANCOVA) design was employed to test for differences in sperm numbers in both species in relation to dose rate, whereby fourth root transformed sperm data was the dependent variable, dose rate was a fixed factor and male weight was used as a covariate. In gammaridean amphipods, weight is an important covariate in sperm count analyses, with a positive correlation between sperm numbers and body weight generally recorded (e.g. Yang et al., 2008). The relationship between arcsine transformed sperm viability (% Live Sperm, Ahrens et al., 1990) and male weight was first analysed using linear regression. A general linear model (GLM) was then performed with dose rate as a fixed factor to determine the impact of radiation on sperm viability for both species. For the *E. marinus* models, exposure (trial 1 or 2) was added as an additional random factor to test for differences in sperm number between the two experiments. All of the above tests were performed in IBM SPSS (v 22.0). Differences in the amount of DNA damage between dose rates was assessed using ANOVA on log-transformed % tail DNA data with the R function aov (R Studio v 1.0136, R Core Development Team, 2016). Assumptions of the analyses were tested by inspection of the residuals and normality testing using the shapiro.test function.

iii) Breeding Experiment

A Kruskal-Wallis H test was used to test for differences in time taken to reproduce between treatments owing to a non-normal distribution of the dataset. Differences in fecundity between females breeding with IR-exposed males were tested using an ANCOVA design with female weight as a covariate, as larger females typically produce larger broods in amphipods (Ford et al., 2003). Differences in embryo diameters and percentage of abnormalities per female were tested using a Kruskal-Wallis H test and a 2 x 4 contingency table with a subsequent χ^2 test respectively.

2.3 Results

2.3.1 Moulting & Mortality

Radiation dose rate did not affect the frequency of moulting in either *E. marinus* (GZLM, df = 3, $\chi^2 = 0.467. = p > 0.05$) or *G. pulex* (GLZM, df = 3, $\chi^2 = 0.931$, $p > 0.05$). In *E. marinus*, both time (df = 13, $\chi^2 = 12.441$, $p < 0.05$) and trial (df = 2, $\chi^2 = 5.351$, $p < 0.05$) had significant effects on moulting, suggesting that the frequency of moulting events varied over the course of the experiments and between individual trials. However, the non-significant interaction term between dose rate and time ($\chi^2 = 0.393$, df = 17, $p > 0.05$) and dose rate and trial ($\chi^2 = 1.569$, df = 4, $p > 0.05$) implied that these differences were unrelated to radiation treatments. In *E. marinus*, average mortality across exposures ranged from 0% in controls to 13.9 ± 10.5% (mean ± standard deviation) in 10 mGy/d treatments. Mortality in *G. pulex* exposures was higher, ranging from an average of 14.6 ± 2.9% in controls to 25 ± 5.9% in 10 mGy/d treated individuals, increasing in a dose-dependent manner. Cumulative mortality for all P-32 exposures pooled are shown in Figure 2.6.

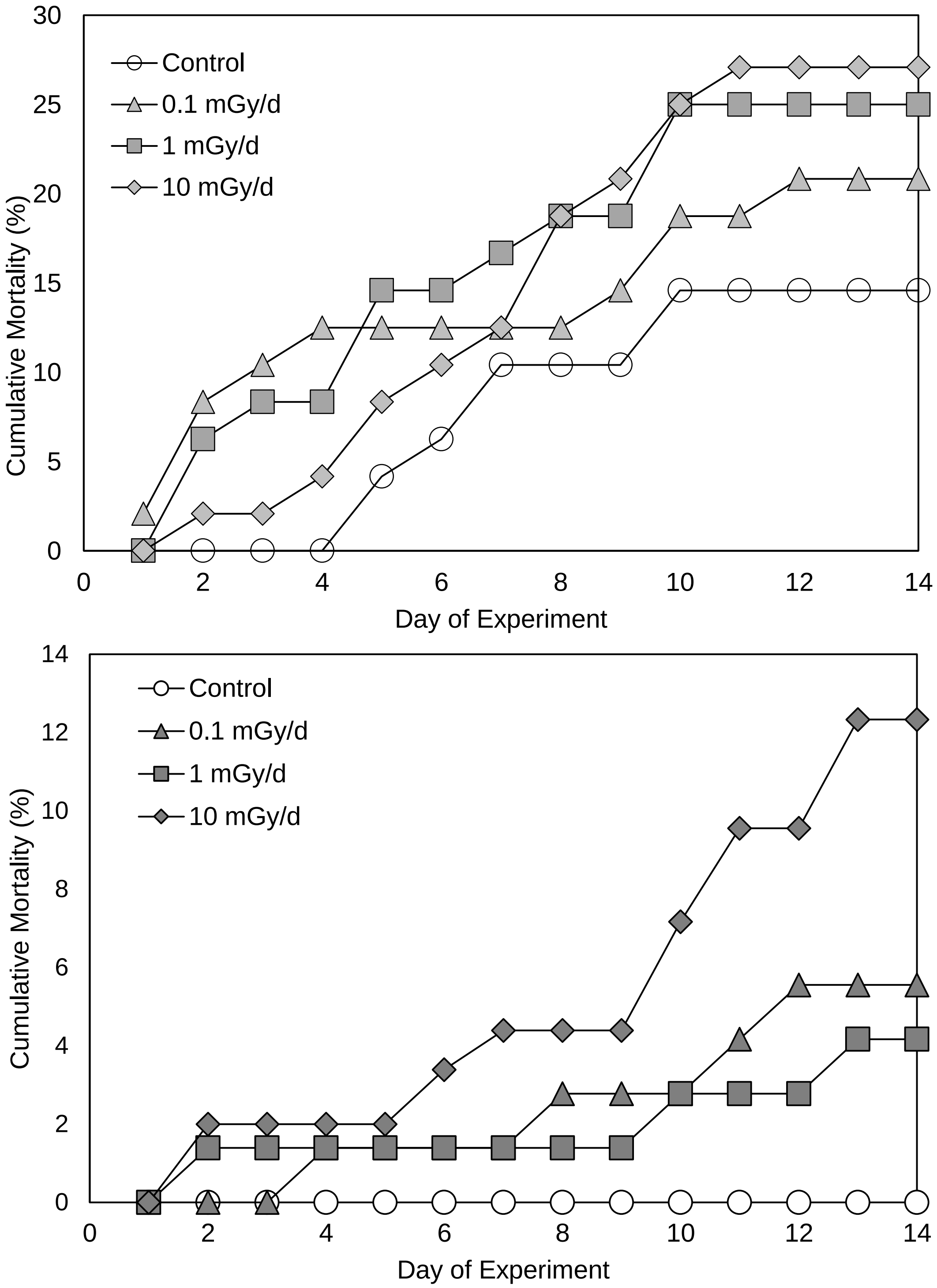


Figure 2.6 – Cumulative mortality in *G. pulex* (Top) and *E. marinus* (Bottom) exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d in the laboratory (n = 24 for both species) for 14 days.

2.3.2 Uptake of ^{32}P by Echinogammarus marinus and Gammarus pulex

i) Echinogammarus marinus

Initial activity concentrations of the exposure medium were 9.58 ± 4.32 Bq/ml (Mean ± standard deviation), close to the expected values of 10 Bq/ml. The mean activity concentration for the exposure period was 8.76 ± 0.60 Bq/ml. Mortality was low, with only one death recorded for the 14 day exposure. Mean activity concentrations were 222.27 ± 78.98, 310.67 ± 161.966 and 293.95 ± 104.79 Bq/g for whole organisms, exoskeleton and hepatopancreatic tissue respectively. No significant differences were recorded in activity concentrations of ^{32}P between whole organisms and other tissues (ANOVA, F = 2.389, df = 2, p = > 0.05), owing to large variability between individuals. Subsequent experiments with *G. pulex* were therefore undertaken using whole individuals only. For the initial uptake experiment, mean CR values were calculated to be 25.66 ± 9.31 l kg^{-1} (N = 9). Pooled with LSC uptake measurements from subsequent fertility, breeding and genotoxicity exposures, a mean *E. marinus* CR value of 33.18 ± 6.21 l kg^{-1} (N = 41) was calculated (see Figure 2.7).

ii) Gammarus pulex

Initial activity concentrations for the *G. pulex* exposures were 5.71 ± 1.12 Bq/ml, where expected activity concentrations were 5 Bq/ml. Mean activity concentrations over the exposure period were 5.98 ± 0.61 Bq/ml. Mortality was low, with only one death recorded during the 14 day exposure period. Mean activity concentrations for whole organisms were 1330.87 ± 670.86 Bq/g, leading to a CR value of 275.73 ± 126.50 l kg^{-1} (N = 9). For pooled uptake measurements with subsequent experiments, a CR value of 174. 76 ± 49.89 l kg^{-1} was derived (N = 51). Significant differences (Kruskal Wallis H-Test, χ^2 = 50. 76. df = 1, p = < 0.05) in overall CR values were observed between the two species, with CR values for *G. pulex* 5-fold greater than those in *E. marinus* (see Figure 2.7).

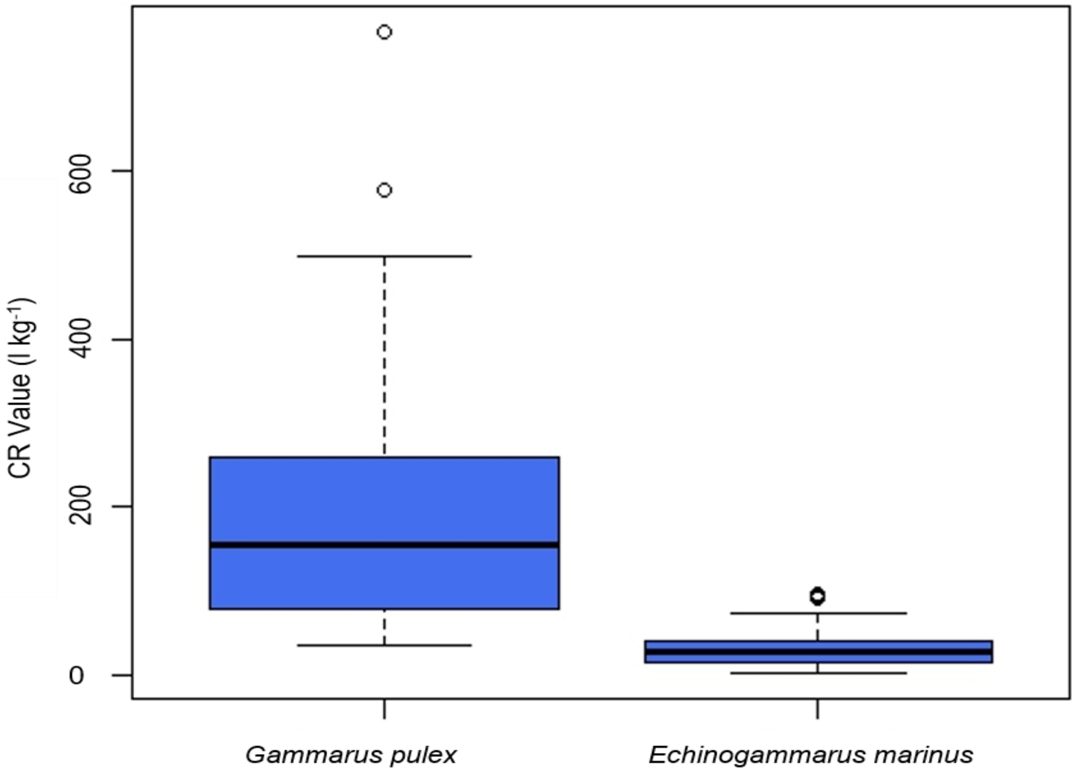


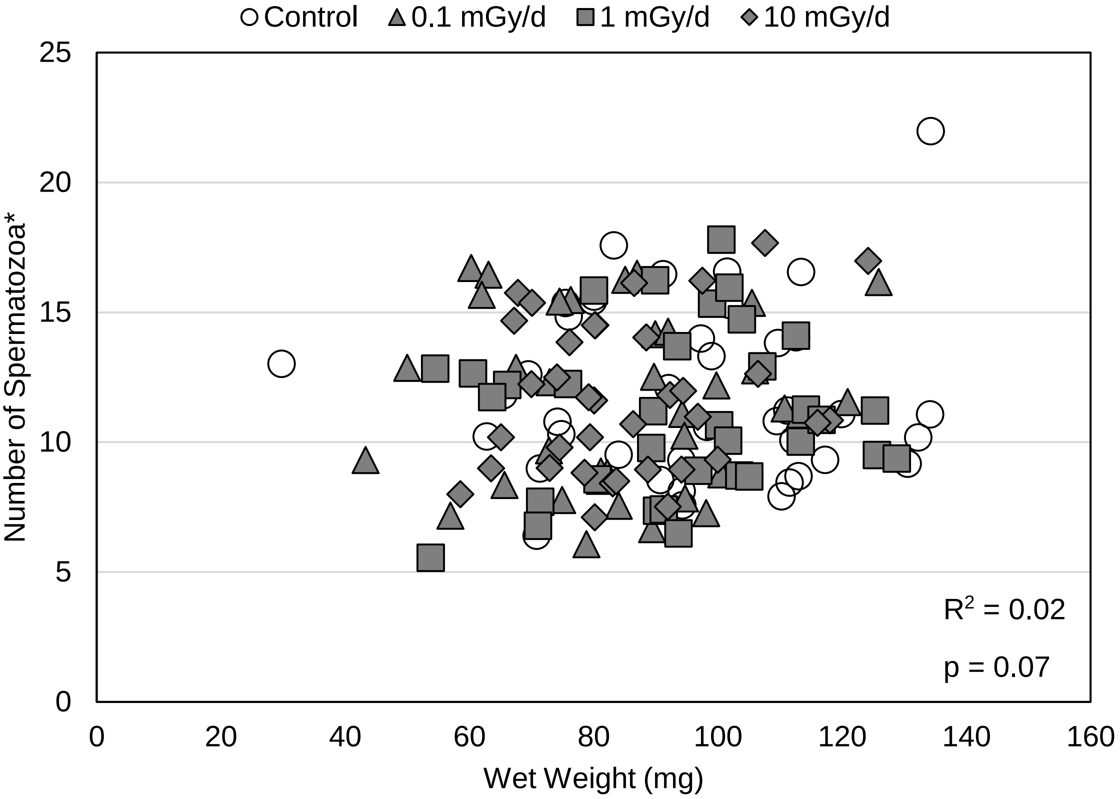
Figure 2.7 – Boxplot of concentration ratio values for phosphorus-32 in *Echinogammarus marinus* and *Gammarus pulex.*

2.3.3 Effects of Ionising Radiation on Male Fertility

I) Sperm Counts

Radiation dose rate did not affect the numbers of sperm produced by *E. marinus* (see Figure 2.9, $F_{1, 136}$ =1.423, df = 3, $p > 0.05$). Mean sperm numbers over the two exposures were 17,919 ± 100 or 11.57 ± 3.17 following fourth root transformation (N = 148). Significant differences in sperm numbers were observed between the two trials (ANCOVA, $F_{1, 136}$ = 193, $p < 0.05$) with 9.25 ± 1.59 and 13.95 ± 2.56 recorded in trials 1 and 2 respectively. However, a non-significant interaction term between radiation dose rate and trial ($F_{3, 136}$ = 1.173, $p > 0.05$) suggested the effect of radiation was consistent between experiments. Wet weight had a highly significant effect on sperm numbers ($F_{1, 136}$ = 40.60, $p < 0.001$).

In *Gammarus pulex*, no significant effect of radiation dose rate on sperm numbers was recorded (see Figure 2.9, $F_{3, 60} = 1.214$, $p > 0.05$). Mean sperm numbers were 3331 ± 2071, or 7.60 ± 6.75 following fourth root transformation (N = 61). Highest sperm numbers were recorded in the control group (8.90 ± 2.65), with individuals in the 1 mGy/d displaying the fewest sperm (5.95 ± 2.17). Wet weight had a highly significant effect on numbers of sperm (see Figure 2.8, $F_{1, 60} = 27.39$, $p < 0.001$).



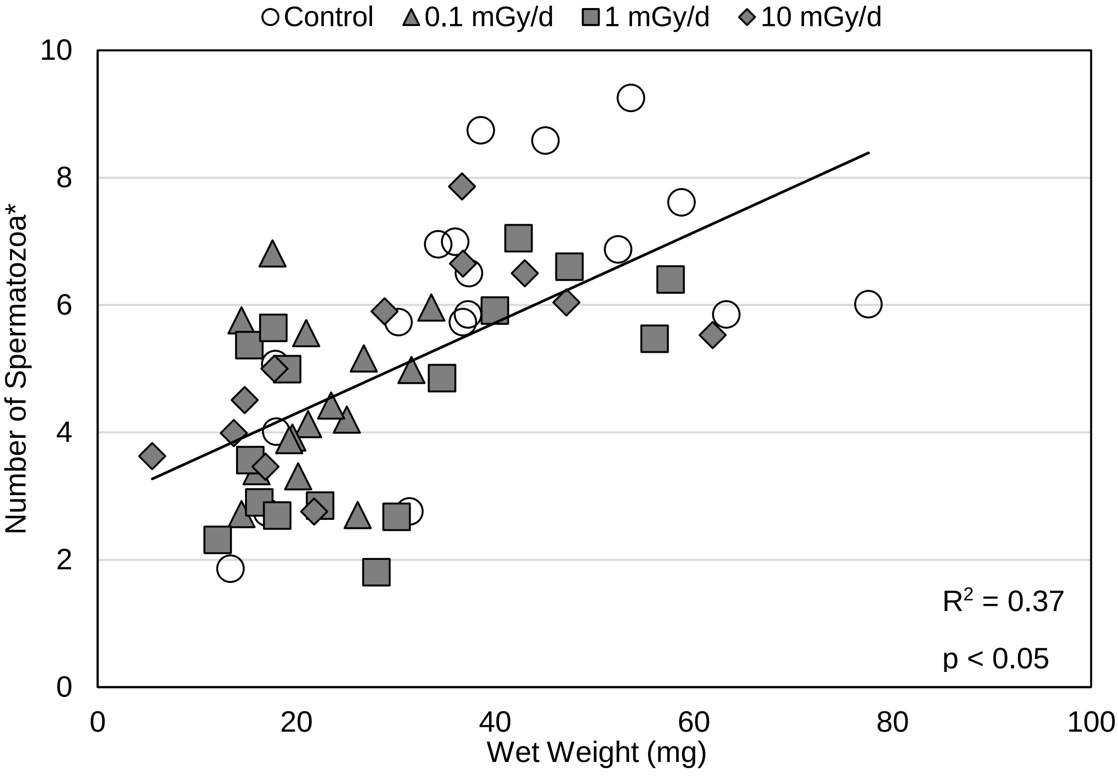
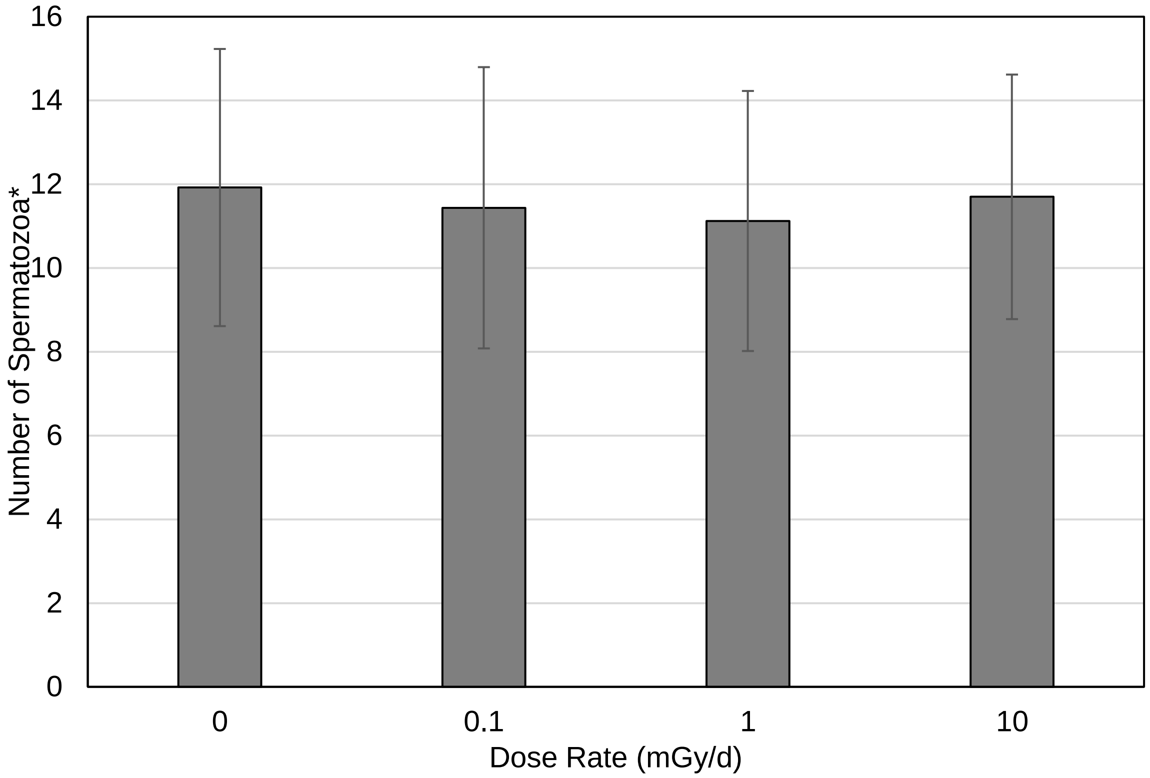


Figure 2.8 – Relationship between wet weight and total numbers of sperm per individual in *Echinogammarus marinus* (top) and *Gammarus pulex* (bottom) exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d in the laboratory. * = Fourth root transformed.



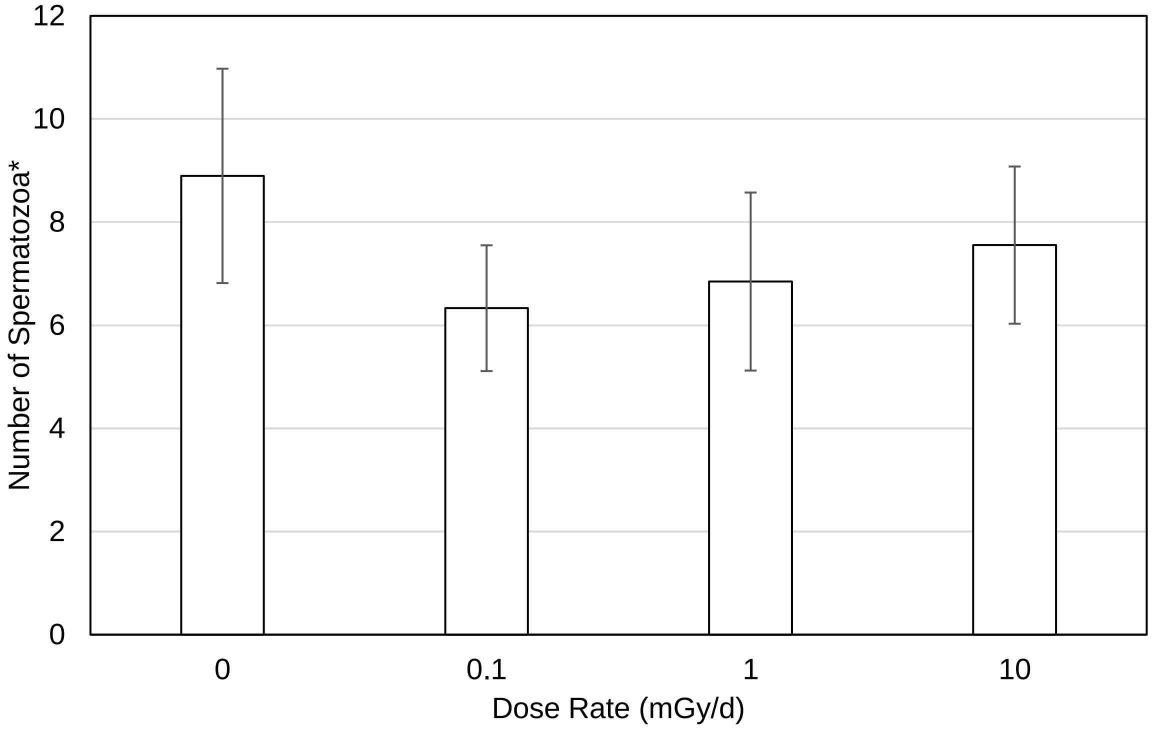


Figure 2.9 – Mean sperm numbers in *Echinogammarus marinus* (top) and *Gammarus pulex* (bottom) exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d in the laboratory (n = 24). * = Fourth root transformed. Error bars are ± Standard Deviation.

II) Sperm Viability

E. marinus sperm viability decreased in a dose dependent manner with increasing radiation dose rate (see Figure 2.10), with significant reductions of 9 and 11% relative to controls at 1 and 10 mGy/d (ANOVA, Post-hoc Dunnett's test, $p < 0.001$). No significant differences were observed between control and 0.1 mGy/d treated groups (Post-hoc Dunnett's test, $p > 0.05$). Mean sperm viability (% Live Sperm) for the two trials was 86.59 ± 9.20%. No significant differences in arcsine transformed viability was recorded between trials (ANOVA, $F_{1, 140} = .707$, $p > 0.05$). Response to radiation was shown to be consistent across both trials owing to a non-significant interaction term between dose rate and trial ($F_{1, 140} = 1.421$, $p > 0.05$). Wet weight was not significantly correlated with sperm viability (Regression analysis, $F_{1, 145} = 3.334$, $R^2 = .022$, $p > 0.05$).

In *Gammarus pulex*, a weak but significant positive relationship between wet weight and arcsine viability was recorded (Regression analysis, $F_{1, 59} = 4.042$, $R^2 = 0.064$, $p < 0.05$) therefore an ANCOVA model was used for subsequent analysis with wet weight as a covariate. Sperm viability was reduced at all dose rates relative to controls, with the lowest sperm viability observed in the 1 mGy/d treatment (69.10 ± 20.47%). However, the effects of radiation on arcsine transformed sperm viability in *G. pulex* were non-significant (ANCOVA, $F_{3, 60} = .883$, $p > 0.05$) owing to high inter-individual variability (see Figure 2.10). Mean sperm viability in *G. pulex* across all treatments was 73.55 ± 16.07%.

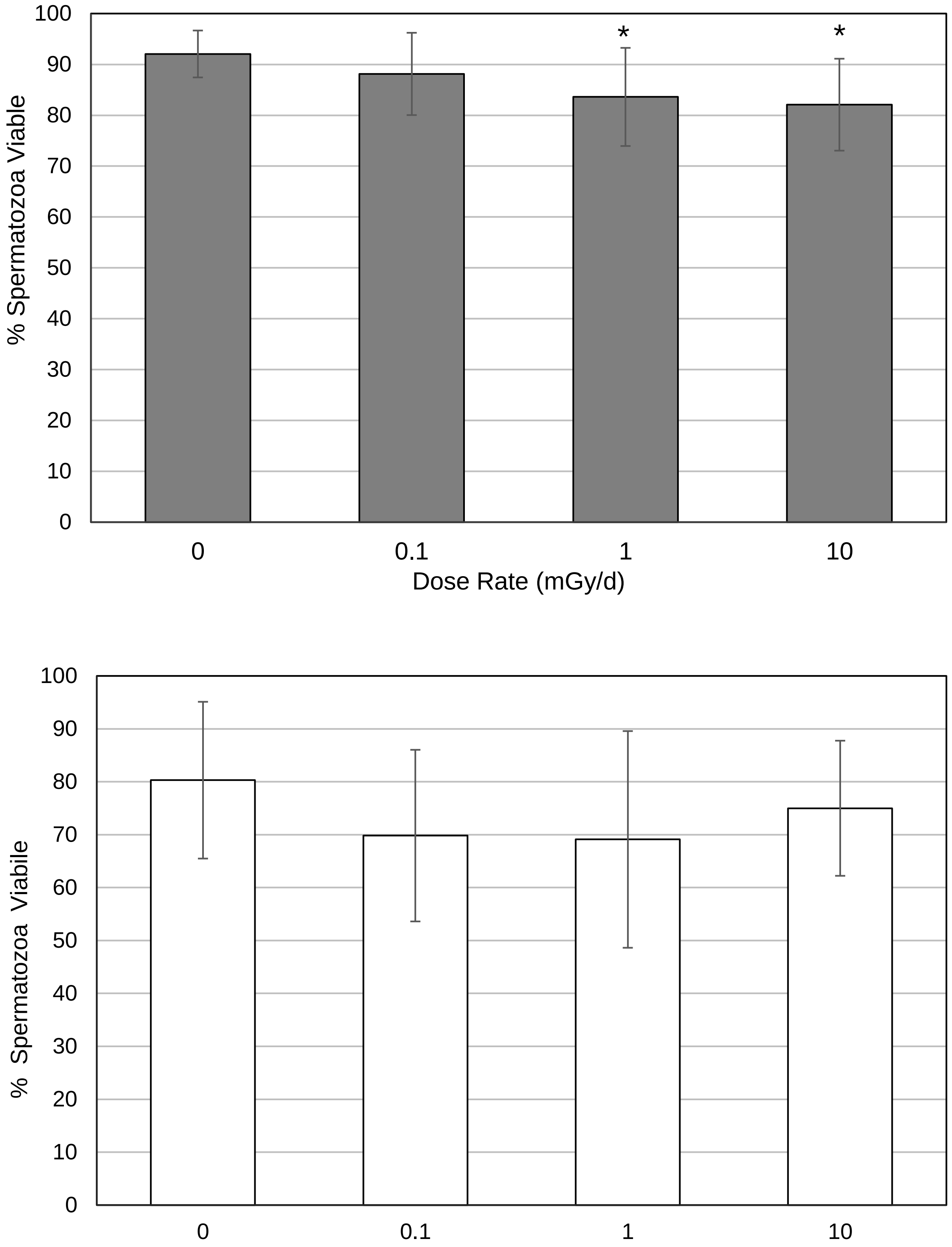


Figure 2.10 – Mean sperm viability in *Echinogammarus marinus* (top) and *Gammarus pulex* (bottom) exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d in the laboratory (n = 24). * Represents significant (Post-hoc Dunnet's Test, $p < 0.05$) difference from control. Error bars are ± Standard Deviation.

2.3.4 Genotoxicity of IR on E. marinus *Sperm Cells*

Radiation dose rate had a significant (ANOVA, $F_{1,\ 66} = 4.987$, $p < 0.05$) effect on DNA damage in *E. marinus* spermatozoa. DNA damage increased with increasing dose rate, with the lowest % tail DNA recorded in the control organisms (10.30 ± 7.51%) and the greatest in the 10 mGy/d treatment (20.69 ± 12.74 %, see Figure 2.11). Subsequent post-hoc pairwise comparisons found significant differences in DNA damage between control and 10 mGy/d treatments only (Pairwise comparisons, Bonferroni correction, $p < 0.05$, see Figure 2.11). A weak, significant positive relationship was recorded between log transformed DNA damage and dose rate ($R^2 = .071$, $F_{1,\ 66} = 4.978$, $p < 0.05$).

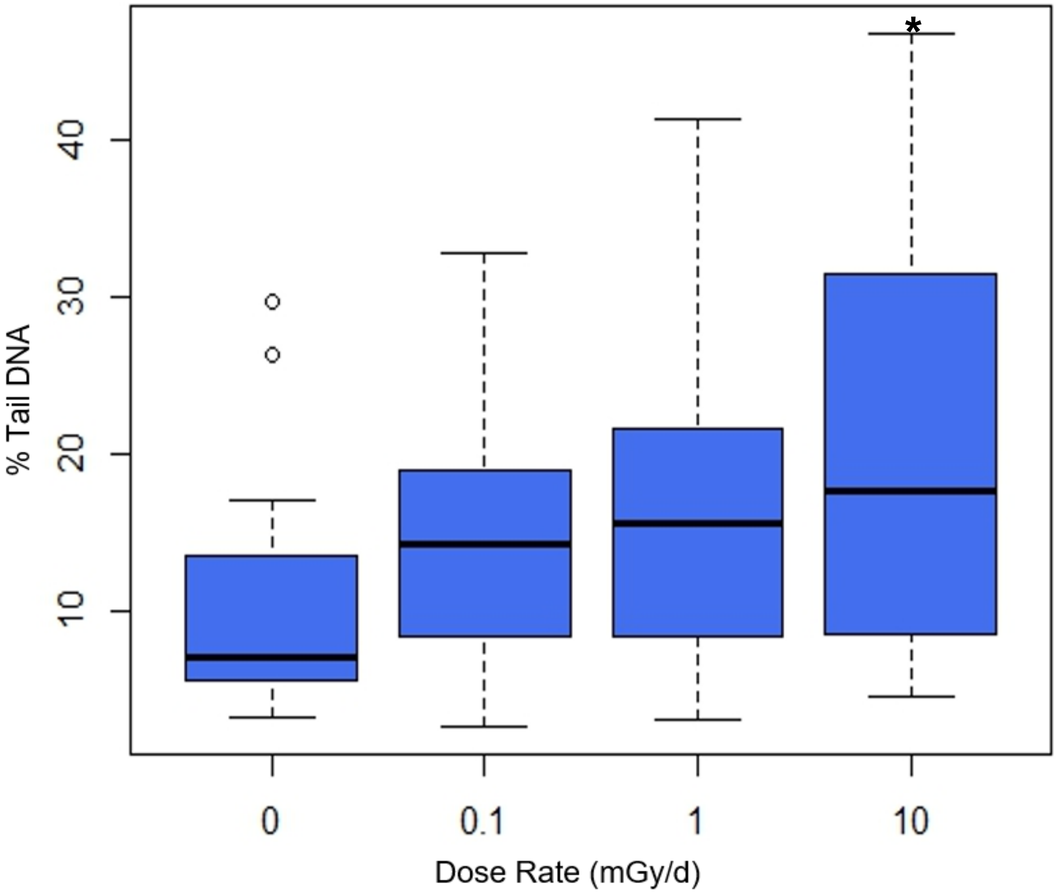


Figure 2.11 – Boxplot of DNA damage (measured as % Tail DNA) in *Echinogammarus marinus* spermatozoa exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d in the laboratory. * Represents significant difference from control (Pairwise comparisons, Bonferroni correction, $p < 0.05$).

2.3.5 Breeding Experiment

i) Time Taken to Reproduce

Mean time taken to reproduce following pairing across all treatments was 14.67 ± 3.91 days. Females breeding with males from the control and 1 mGy/d groups reproduced the fastest, taking on average 11.29 ± 4.59 and 11.81 ± 8.79 days respectively. Individuals in the 0.1 and 10 mGy/d treatments took longer to reproduce (15.93 ± 11.60 and 19.65 ± 10.06 days respectively), though this was non-significant (Kruskal Wallis H-test, $\chi^2 = 7.661$, df = 3, $p = 0.054$). The percentage of breeding females per treatment over time is shown in Figure 2.12.

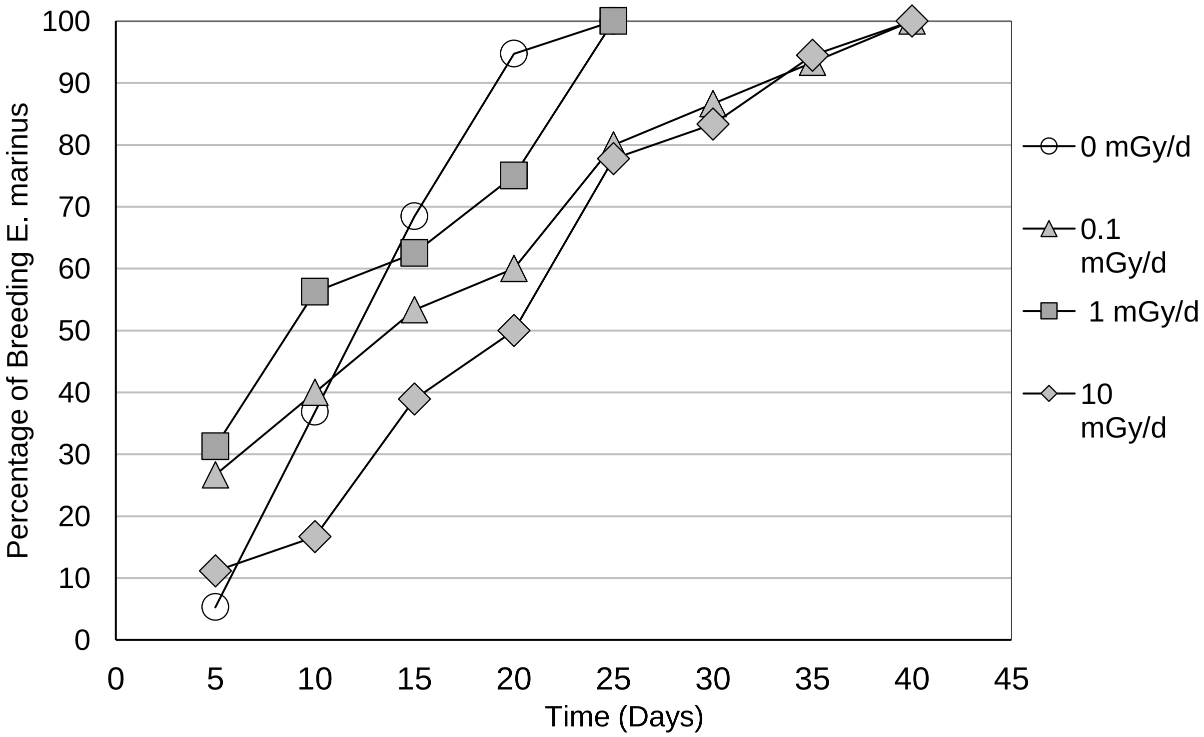


Figure 2.12 – Percentage of non-exposed female *E. marinus* individuals breeding with males exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d over time (n = 20).

ii) Fecundity and Embryo Abnormalities

Female *E. marinus* produced on average 32.37 ± 19.95 eggs across all treatments. Female weight had a highly significant effect on fecundity (ANCOVA, $F_{4, 60} = 4.296$, $p < 0.05$) with a significant positive relationship recorded between female wet weight and number of eggs produced (Regression analysis, $R^2 = 0.09$, $F_{1, 63} = 6.474$, $p < 0.05$). Females breeding with males from the control group produced the greatest number of eggs (41.56 ± 3.39), with the fewest produced by females breeding with 0.1 mGy/d treated animals (28.23 ± 5.86). Fecundity was not related to time taken to reproduce (Regression analysis, $R^2 = .021$, $F_{1, 63} = 2.401$, $p > 0.05$). Male exposure to radiation was found to have a significant effect on fecundity in *E. marinus*, owing to significantly smaller broods at all dose rates relative to controls (see Figure 2.13, ANCOVA, $F_{4, 60} = 4.296$, $p < 0.05$). A weak, significant positive relationship between sperm viability and weight-normalised fecundity was observed (see Figure 2.14, Spearmans rank-order correlation, rho = .280, $p < 0.05$).

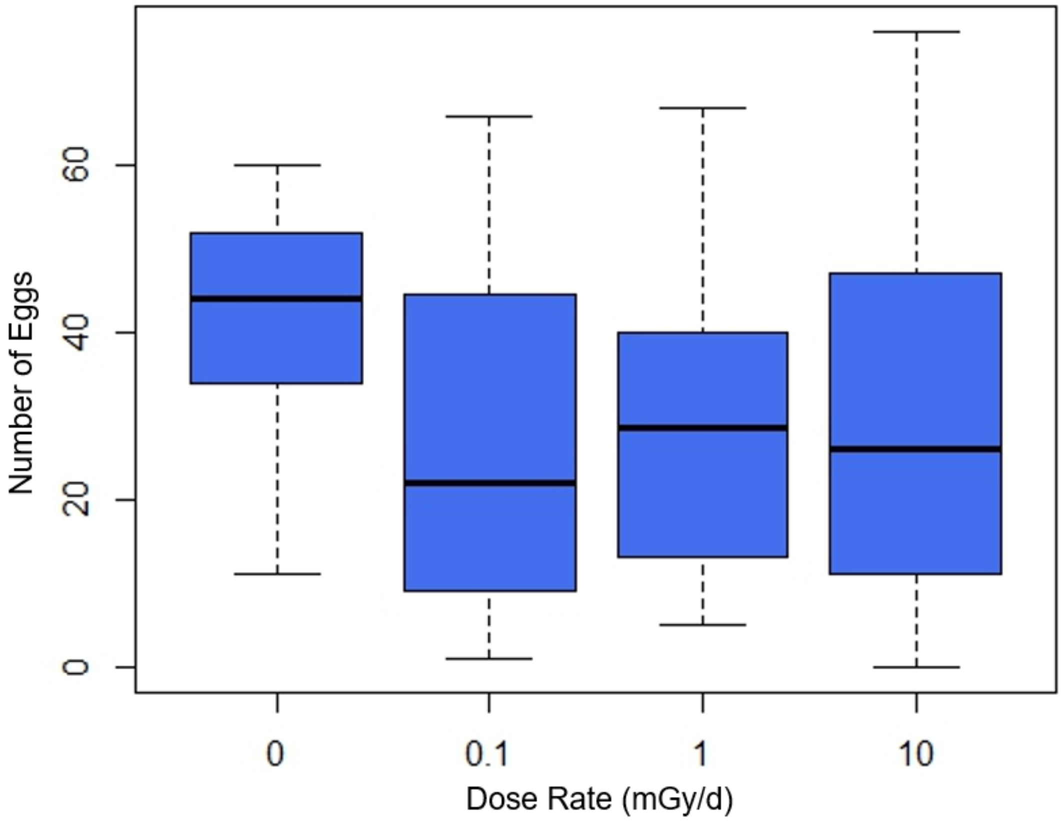


Figure 2.13 – Boxplot of fecundity in *Echinogammarus marinus* females breeding with males exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d (n = 20).

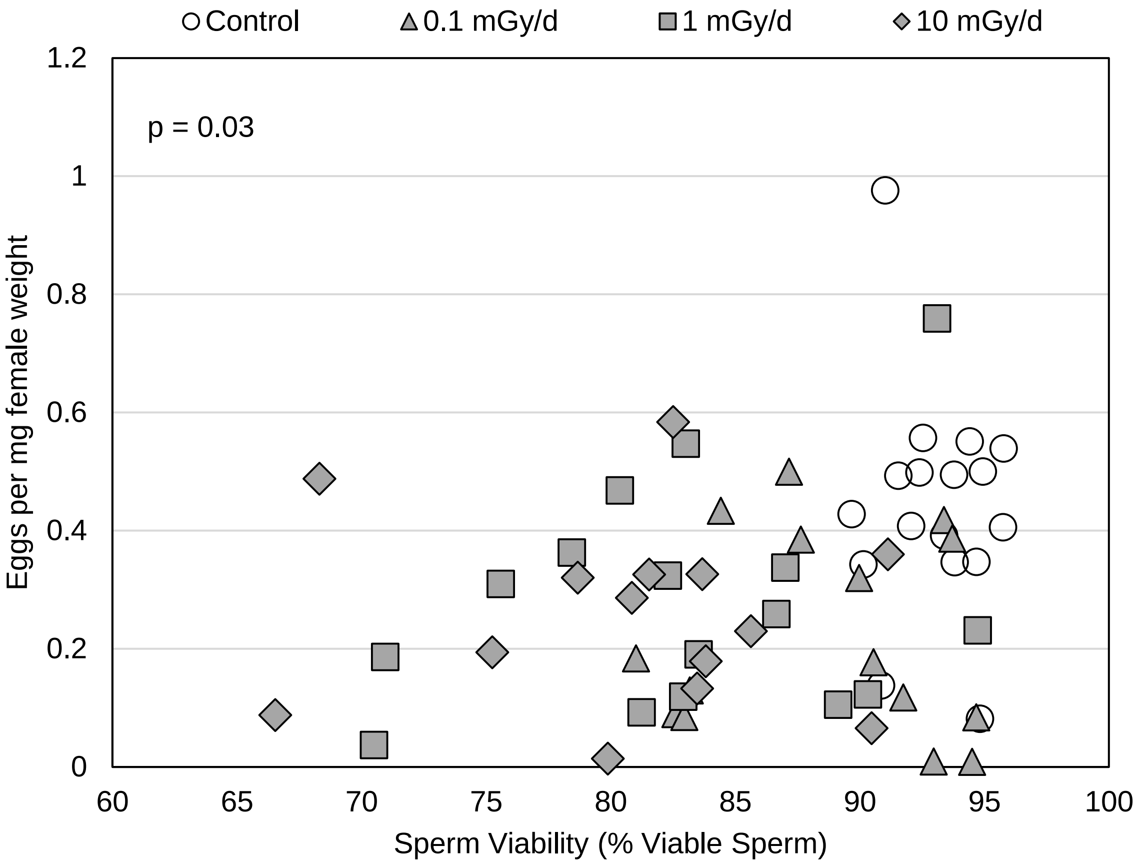


Figure 2.14 – Relationship (Spearmans rho = .280, p < 0 .05) between sperm viability in *E. marinus* exposed to 0, 0.1 and 10 mGy/d from phosphorus-32 in the laboratory and resultant weight-normalised brood sizes following reproduction with a non-exposed female.

Embryo abnormalities per female ranged from 1.9 ± 3.30 % in the control group to a maximum of 17 ± 30.35 % in the 1mGy/d exposed group (see Figure 2.15). This difference was largely attributed to the presence of 'dead' broods wherein all embryos within the brood were abnormal in the 1 and 10 mGy/d treatments (100% embryo abnormality). The frequency of abnormalities per female was significantly increased as compared to the control at both 1 and 10 mGy/d (2 x 4 Contingency Table, $\chi^2 = 13.085$, df = 1, $p < 0.05$ and $\chi^2 = 11.966$, df = 1, $p < 0.05$ respectively). To assess the contribution of dead broods to the observed relationship, the data was also analysed following exclusion of samples exhibiting 100% embryo abnormalities. In this case, a significant increase in abnormalities relative to controls was observed only between 0 and 1 mGy/d, with no significant differences between any other treatments. A weak but significant negative relationship was recorded between sperm DNA damage and the % of embryos developing normally (see Figure 2.16, Spearmans rho = -.310, $p < 0.05$).

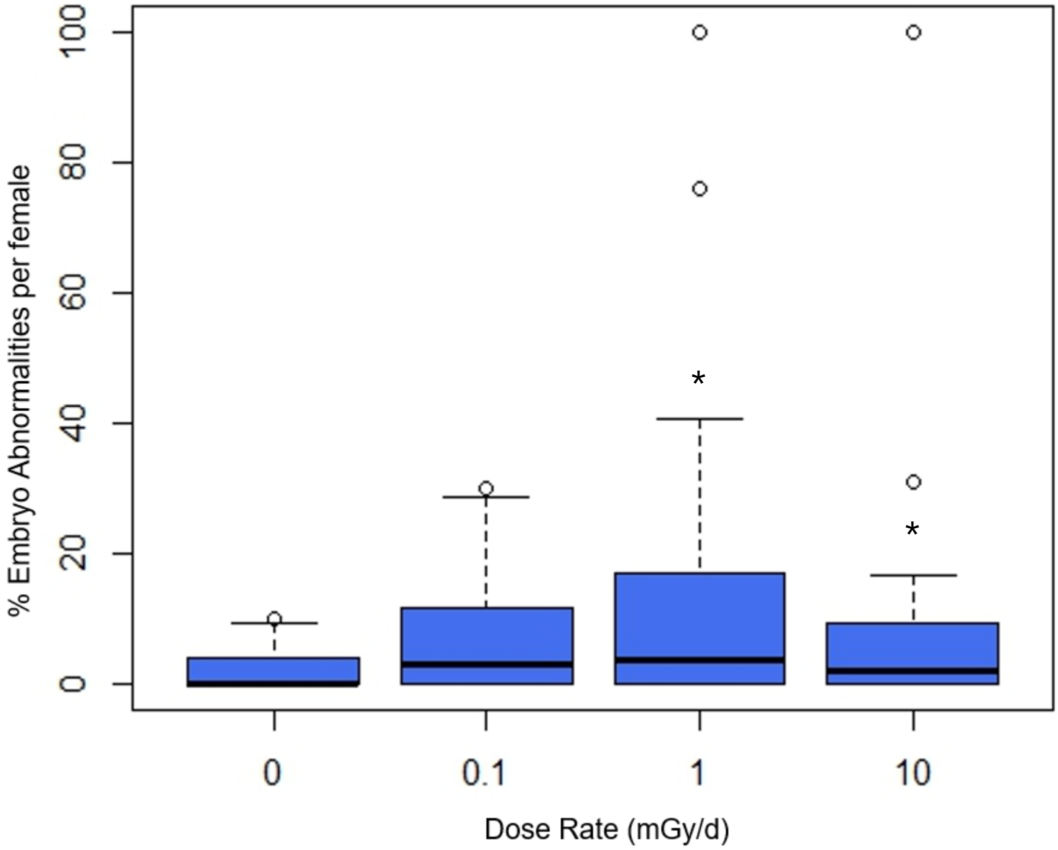


Figure 2.15 – Percentage of abnormal embryos (total number of malformed embryos/total number of embryos per female, Camus & Olsen, 2008) in female *E. marinus* breeding with males exposed to phosphorus-32 at dose rates of 0, 0.1, 1 and 10 mGy/d . * Represents significant difference from control (2 x 4 contingency table, χ^2 test, $p < 0.05$).

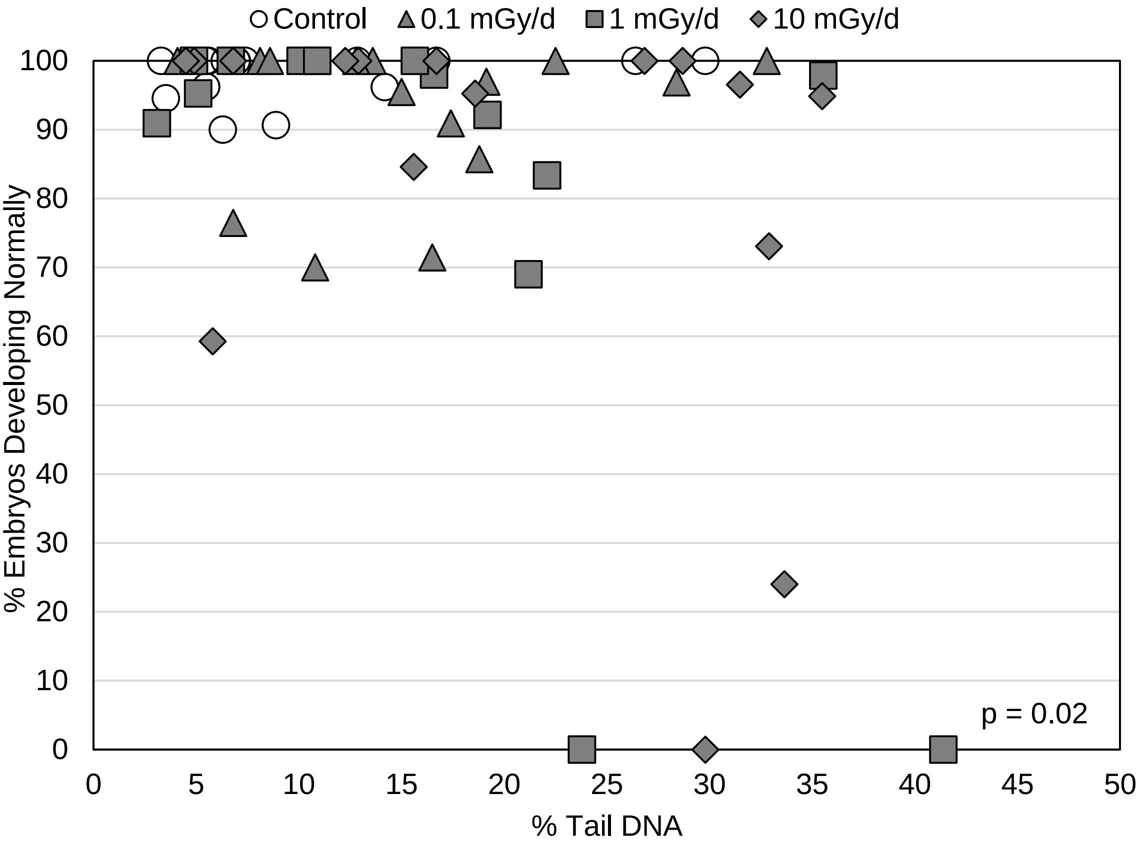


Figure 2.16– Relationship (Spearmans rho = -.310, $p < 0.05$) between DNA damage in the sperm of *Echinogammarus marinus* exposed to phosphorus-32 and development in resultant embryos following reproduction.

2.4 Discussion

2.4.1 Uptake of ^{32}P by E. marinus and G. pulex

Within the ERICA tool, CR values for ^{32}P in marine and freshwater crustaceans are 99000 and 60000 l kg^{-1} respectively (Hosseini et al., 2008). These values are generated from assumptions based on fish and gastropods, and have not been determined experimentally (Yankovich et al., 2013). The present study represents the first experimental derivation of CR values for ^{32}P in marine and freshwater crustaceans. Accumulation of ^{32}P was found to be orders of magnitude lower than predicted based on the values in ERICA, being 33.18 ± 6.21 and 174. 76 ± 49.89 l kg^{-1} for *E. marinus* and *G. pulex* respectively. One possible explanation for the lower CR values may be owing to the experimental design. Organisms were exposed to ^{32}P directly through seawater rather than contaminated feed. A number of studies have demonstrated differences in biokinetics and uptake of radionuclides with exposure pathway. For example, Carvalho & Fowler (1993) exposed the benthic shrimp, *Lysmata seticaudata*, to two radionuclides (Lead-210 and Polonium-210) and found that polonium was largely accumulated from contaminated feed, whereas lead was taken up primarily from seawater. However, a number of other studies of radionuclide uptake in aquatic invertebrates have documented no difference between exposure pathways (Renfro et al., 1975) or greater uptake via the seawater pathway (Warnau et al., 1996,1999). Clearly, radionuclide uptake dynamics depend on a host of physiochemical and ecological factors including the specific radionuclide studied. Future studies should determine the importance of the dietary pathway in uptake of radiophosphorus to crustaceans to assess the appropriateness of the CR values derived in the present study.

In the present study, the freshwater amphipod *G. pulex* was found to have significantly greater uptake and accumulation of ^{32}P as opposed to the marine amphipod *E. marinus*. This could be explained by differences in phosphorus availability between their respective habitats. Traditionally, phosphorus limitation is more prevalent in freshwater systems as opposed to coastal environments wherein nitrogen limitation is more common (Burson et al., 2016). Assuming the environment of *G. pulex* to be more phosphorus limited than that of *E. marinus*, *G. pulex* may have accumulated radioactive phosphorus more readily thus leading to a higher CR value. The CR values determined for *G. pulex* in the present study are within the range of biological concentration factors (BCF) for other pollutants. For example, Miller et al (2016) recorded BCF values ranging from 12 – 4533 l kg^{-1} d^{-1} in *G. pulex* exposed to eight pharmaceutical compounds. In a study of trace metal accumulation in *E. marinus* by Clason et al., (2004), lower BCF values of 33 – 963 were recorded. The authors noted that such values were significantly lower than those of other amphipod species. This may explain the lower CR values for *E. marinus* as compared to *G. pulex* in the present study.

For uptake experiments in *Echinogammarus marinus* and *Gammarus pulex*, individuals were kept at 10 and 15 degrees respectively (see Section 2.2.2) in keeping with previous work on these organisms (Bossus et al., 2014; De Castro-Català et al., 2017; Guler & Ford, 2010). A range of physio-chemical parameters are known to influence radionuclide uptake in organisms including pH, cation exchange capacity and temperature (Vives i Batlle, 2014; Wood et al., 2013). Studies have shown lower radionuclide concentration factors in a range of freshwater and marine organisms at lower temperatures (e.g. Boisson et al., 1997; Nakahara et al., 1977). Therefore, the experimental conditions used may have contributed to the observed differences in CR values between species.

2.4.2 Effects of IR on Male Fertility in E. marinus and *G. pulex*

The present study hypothesised that IR would cause a reduction in both the number and quality of sperm in *E. marinus* and *G. pulex*. In *E. marinus*, a significant reduction in sperm quality at 1 and 10 mGy/d was recorded, though sperm numbers were unaffected. Conversely for the freshwater amphipod *G. pulex*, no effect of radiation on either sperm quality or quantity was recorded. Support for the hypothesis was found for *E. marinus*, but not for *G. pulex*.

At present, three studies have monitored the effects of pollution on male fertility in amphipods (Gismondi et al., 2017; Trapp et al., 2015; Yang et al., 2008). All of these studies recorded significant reductions in the number of spermatozoa in response to industrial pollution, heavy metals (Cadmium) and the endocrine disruptors, pyriproxyfen and cyproterone acetate. Trapp et al., (2015) demonstrated drastic dose-dependent reductions of up to 73 and 75% in the number of spermatozoa in response to cadmium and pyriproxyfen respectively, whilst no effect on the number of spermatids (earlier stage sperm cells) was found. Yang et al., (2008) studied organisms collected from industrially polluted sites. Such organisms would have been exposed to xenobiotics throughout their entire life cycle, as opposed to laboratory studies where lifetime exposures are not commonly used. The difference in exposure duration may account for the differences between studies, though both Trapp et al., (2015) and Gismondi et al., (2017) used similar durations to the present study (15 and 6 days respectively).

One further explanation for the different response in the present study may be owing to the specific pollutants and doses used. Endocrine disruptors such as pyriproxyfen and cyproterone acetate have been shown to have deleterious impacts on the reproductive system of crustaceans at low doses (e.g. Ginjupalli & Baldwin, 2013) due to their role as juvenile hormone analogs. Similarly cadmium is an extremely potent toxicant, with concentrations causing a significant reduction in sperm counts in the previous study of *G. fossarum* spanning an environmentally relevant range of freshwater systems in Europe (Pan et al., 2010). Comparatively, the majority of studies recording a reduced sperm count in response to radiation exposure used acute doses orders of magnitude higher than in the present study (Centola et al., 1994; Rowley et al., 1974).

A number of studies have demonstrated effects of radiation exposure on male fertility in biota at lower doses. Knowles (1999) exposed plaice, *Pleuronectes platessa*, to gamma radiation from ^{137}Cs for 197 days and recorded a reduction in the number of sperm at doses as low as 5.8 mGy/d, lower than the highest dose rate of 10 mGy/d in the present study. Similarly a number of studies have recorded effects on both sperm quantity and quality in birds at Chernobyl at dose rates spanning the range used in the present study (Bonisoli-Alquati et al., 2011; Møller et al., 2014; but see Beresford et al., 2012; Smith, 2008). Radiosensitivity is thought to increase with biological complexity (Hinton et al., 2007), therefore fish and birds would be anticipated to display deleterious effects at doses below less sensitive biota such as aquatic invertebrates. This may account for the lack of an effect on sperm numbers in *E. marinus* and *G. pulex* in the present study.

In *E. marinus*, a significant negative effect on the quality of sperm was observed at 1 and 10 mGy/d. In *G. pulex*, a reduction in the quality of sperm was observed at all dose rates relative to control, though no significant differences were observed owing to high inter-individual variability. One possible explanation for the effect on sperm quality but not sperm quantity may be due to the nature of the specific pollutant and the technique used to assess sperm quality. The LIVE/DEAD viability assay assesses sperm plasma membrane integrity, which is known to be sensitive to ROS that are generated indirectly following radiation exposure. Sperm plasma membranes are rich in polyunsaturated fatty acids (Cerolini et al., 2000; Duru et al., 2000) which are susceptible to lipid peroxidation (oxidative degradation of lipids), leading to loss of membrane integrity. Conversely, the mechanisms reported for reduced sperm counts following irradiation are generally associated with direct cell lethality at acute doses higher than those used in the present study (Ogilvy-Stuart & Shalet, 1993). The present study suggests that sperm quality indicators may be more sensitive indicators of chronic low-dose radiation exposure as opposed to sperm count analyses in amphipods.

2.4.3 Effects of Radiation on DNA Damage in Sperm

It was hypothesised that a reduction in sperm quantity and quality caused by ionising radiation would be linked to an increase in sperm DNA damage. Due to high mortality in control cultures of *Gammarus pulex* under experimental conditions (see Sect 2.3.1), this was only assessed for *E. marinus*. In the present study, the amount of sperm DNA damage increased with radiation dose rate, thereby supporting the hypothesis. However, a significant elevation in genotoxicity was only observed in the 10 mGy/d treated group relative to controls. Despite a logarithmic increase in dose rate, mean DNA damage did not increase concomitantly, suggesting a non-linear dose response. This may be owing to elevated DNA repair activity at higher dose rates. It has been hypothesised that a threshold exists for radiation-induced activation of DNA repair, which has been demonstrated in fish (Grygoryev et al., 2013). Similarly, induction of DNA repair following radiation exposure has been observed in copepods exposed to acute high doses of gamma radiation (e.g. Han et al., 2014a). Therefore, a threshold may exist in *E. marinus* above which DNA repair is stimulated, as proposed for model fish species.

In spermatozoa however, mammalian studies have demonstrated reduced DNA repair capacity (Aitken & De Iuliis, 2007). During the late phase of spermatogenesis, DNA repair is down-regulated leading to reduced repair capabilities in sperm (Lewis & Aitken, 2005). In amphipods, Lacaze et al., (2011) found no reduction in DNA damage in *Gammarus fossarum* sperm 4 days post exposure to genotoxicants, suggesting limited DNA repair capacity. Conversely, Lewis & Galloway (2009) demonstrated recovery of sperm cells in *Mytilus edulis* 72 hours post-exposure to MMS, though recovery was lower than in somatic cell types. No studies have assessed sperm DNA damage repair in aquatic invertebrates exposed to ionising radiation. Given the unique signature of DNA damage induced by IR (see Sect 2.1.2), further studies of DNA repair in aquatic invertebrate germ cells are necessary to contextualise the findings of the present study.

2.4.4 Knock-on Effects on Breeding

In the present study, radiation exposure of male *E. marinus* had a significant effect on both the number and quality of resultant offspring following breeding (see Sect 2.3.5). A delay in reproduction was also observed in males exposed to 10 mGy/d, however this was non-significant. This suggests that perturbations to male fertility, e.g. a reduction in sperm quality may have concomitant effects of female reproductive output in amphipods. Sperm quality has been demonstrated to be a key determinant of fertilization success in a range of aquatic organisms including fish (Casselman et al., 2006), urchins (Au et al., 2001) and polychaete worms (Lewis et al., 2008). In amphipod crustaceans, the potential for reductions in sperm count to have drastic effects on resultant fecundity has been highlighted by Ford et al., (2012) and is explained in greater detail in Section 1.3.4. To the author's knowledge, no study has assessed the consequences of perturbations to sperm quality on resultant reproductive success in crustaceans.

A weak positive relationship between sperm viability and resultant brood sizes in females was demonstrated (see Figure 2.14), suggesting that perturbations to sperm quality may have been the mechanism for the reduced fecundity observed in females breeding with males exposed to IR. However, the magnitude of the effect on fecundity was much greater than those observed for sperm quality, suggesting that another factor may have contributed to the effects on breeding. For example, significant reductions in brood sizes in females breeding with males exposed to 0.1 mGy/d were observed, wherein only subtle non-significant effects (4% reduction in sperm quality) were observed. In *E. marinus*, brood sizes typically range from 11 – 40 eggs (Cheng, 1942) and vary significantly even within females of the same size class. The results of the present study were characterised by high inter-individual variability, especially in radiation exposed groups (see Figure 2.13), which may have obscured any dose-response relationships. Given the limited sample size (N = 66) and high inter-individual variability, a repeat of the present experiment is necessary to confirm the observed effects on breeding.

Following reproduction with males exposed to 1 and 10 mGy/d, a greater frequency of embryo abnormalities were observed. A negative relationship between sperm DNA damage and the percentage of normally developing embryos was recorded (see Figure 2.16), suggesting that paternal DNA damage may have led to perturbations to early development of offspring. This has been documented for other aquatic invertebrates exposed to genotoxicants. Lewis & Galloway (2009) exposed *Arenicola marina* and *Mytilus edulis* to the model DNA-damaging chemicals MMS and benzo(a)pyrene and monitored sperm DNA damage and resultant impacts on breeding. No impact of sperm DNA damage on fertilization success was observed for either species, though significant effects on post-fertilization development of offspring was observed in both species. Similar results have been demonstrated in studies on fish sperm exposed to MMS, with no influence of paternal exposure on fertilization success but significant effects on abnormalities in progeny (Devaux et al., 2011). In bovine sperm exposed to x-rays and gamma radiation, increased sperm DNA damage did not impair fertilization success but led to perturbations in resultant embryonic development (Fatehi, 2006). Germ cell mutations are thought to persist in subsequent embryonic cell generations, leading to transmission of paternal genetic damage (Luke et al., 1997). This phenomenon may explain the relationship between sperm DNA damage and frequency of embryonic abnormalities observed in the present study. Given the limited sample size and the disproportionate contribution of organisms exhibiting entirely abnormal broods to the observed relationship, (see Section 2.3.5ii) a repeat of the experiment would be necessary to confirm this result.

2.5 Conclusions

This study is the first to assess the effects of IR on male fertility and sperm DNA damage in an aquatic invertebrate. In the marine amphipod, *Echinogammarus marinus*, significant effects of IR exposure on sperm quality but not quantity were found. Perturbations to sperm quality were associated with a reduction in resultant female brood sizes following reproduction. An increase in sperm DNA damage in *E. marinus* was found, which was related to an increase in developmental abnormalities in resultant offspring. Paternal transmission of genetic damage to offspring was proposed as the mechanism. Significant effects on *E. marinus* sperm quality were detected at dose rates of 1 mGy/d (41.7 µGy/hr), below proposed benchmarks wherein negative effects on crustaceans are anticipated (e.g. ICRP, 2008). This suggests that sperm quality may be used as a sensitive endpoint of radiation exposure in crustaceans and that current environmental radioprotection benchmarks may be insufficient to provide protection for all species. However, the majority of environmental protection benchmarks refer to population level effects. Future research should therefore focus on directly linking the effects observed in the present study to higher-level consequences that are the goal of environmental protection.

In the freshwater amphipod, *Gammarus pulex*, no significant effects on either sperm quality or quantity were found. This suggests that *G. pulex* sperm was less sensitive to radiation exposure as compared to the marine *E. marinus*. In *G. pulex*, sperm numbers are approximately 10,000 per testis (Lemaître et al., 2009) and were found to be significantly lower as compared to *E. marinus* in the present study (see Sect 2.3.3i). Given the lower sperm numbers, high reproductive investment and sperm competition recorded previously in *G. pulex* (Lemaître et al., 2009), this species may be under greater selective pressure to maintain sperm quality following xenobiotic exposure as compared to *Echinogammarus* *marinus*. However, further research of the extent of sperm competition and reproductive biology of *E. marinus* would be necessary to understand the drivers of the differences in sensitivity.

Chapter 3

Effects of Chronic Radiation Exposure on Developmental Stability of Crustaceans at Chernobyl and Fukushima

Published in part as Fuller et al., (2017) *Sci. Total. Environ. 576:242-250. (See Appendix B).*

3.1 Introduction

3.1.1 What is Fluctuating Asymmetry?

The developmental stability of an organism is reflected in its ability to present an optimal phenotype under a given set of environmental conditions (Palmer, 1994). Bilaterally symmetrical structures offer a precise, ideal form against which departures can be measured. Fluctuating asymmetry (FA) refers to subtle, random deviations from the expected perfect bilateral symmetry displaying a normal distribution with a mean of zero. Deviations from bilateral symmetry are most commonly described by frequency distributions of right minus left (R-L). Frequency distributions normally conform to one of three patterns; fluctuating asymmetry, antisymmetry or directional asymmetry (DA, See Figure 3.1). Antisymmetry refers to a pattern of bilateral variation wherein significant R-L differences exist, displaying a bimodal distribution with a mean either side of zero (Fig 3.1C, Palmer & Strobeck, 1986). The side of the organism presenting differences varies randomly between individuals. Since one of the fundamental assumptions of FA is normality of R-L distributions owing to subtle, random processes affecting both sides independently, antisymmetry is not typically used as an indicator of developmental stability. Directional asymmetry (Figure 3.1B) occurs wherein one side of an organism is always larger than the other, with the larger side being the same between individuals. Since this form of asymmetry may have a heritable basis and therefore not be indicative of developmental stress, traits exhibiting directional asymmetry are not used for studies of FA. The frequency distribution for typical FA is shown in Figure 3.1A.

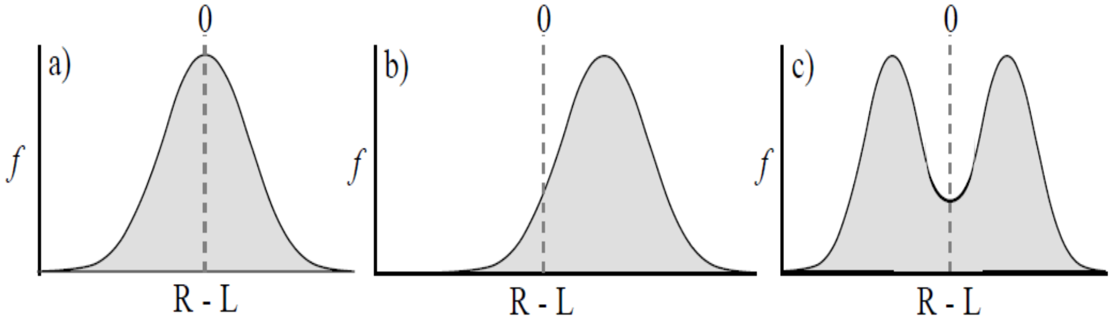


Figure 3.1 – Common frequency distributions of right minus left differences in bilaterally symmetrical organisms. Zero represents the expected 'perfect' bilateral symmetry. a) Fluctuating asymmetry b) Directional asymmetry and c) Antisymmetry. From Palmer (1994).

3.1.2 Fluctuating Asymmetry in Relation to Stress & Fitness

An increase in FA has been recorded in response to a range of extrinsic and intrinsic stressors such as organic pollutants (Jenssen et al., 2010) temperature (Vishalakshi & Singh, 2008) and genetic stressors such as inbreeding (Özener, 2010). Elevated energy expenditure under stressful conditions and a subsequent reduction in available energy for maintaining developmental stability is one possible mechanism for the observed positive relationship between stress and FA. A meta-analysis of 53 studies on the use of FA as a biomarker of environmental stress in insects concluded that FA is a sensitive indicator of stress (Beasley et al., 2013). However, the relationship between stress and FA is not ubiquitous with a number of studies reporting no increase in FA with stress (Bjorksten et al., 2000; Floate & Fox, 2000).

Various studies have attempted to link increases in FA with traditional measures of fitness such as reproductive success and offspring viability. In a study of the three-spined stickleback, *Gasterosteus aculeatus*, male sticklebacks exhibiting lower degrees of asymmetry in ventral spines had greater reproductive success (Bakker et al., 2006). Similarly, Naugler & Leech (1994) recorded reduced survival in individuals of the forest tent caterpillar moth, *Malacosoma disstria* that exhibited a greater degree of asymmetry.

However the relationship between FA and fitness is highly controversial, owing to a body of evidence suggesting that FA cannot be used to reliably indicate fitness (see Lens et al., 2002 for summary). For example, fluctuating asymmetry in the nectar guide of the Andean monkey flower, *Mimulus luteus,* was not found to be related to female fitness (Botto-Mahan et al., 2004). Similarly in an experimental analysis of the relationship between FA and fitness in *Drosophila melanogaster*, Bourguet (2000) found no relationship between FA and two measures of fitness. Some authors have argued that trait-fitness relationships are character specific (Clarke, 2003) and that asymmetry in sexual characters may reliably indicate male quality (Dongen, 2006). This is thought to be owing to elevated production costs in developing elaborate sexual characters and the strong associated directional selection which may select against modifiers that buffer development (Manning & Chamberlain, 1994).

Consequently, many studies of FA have focused on sexual traits and relationships between environmental stress and developmental instability. For example (Møller, 1994) measured FA in a range of traits in the barn swallow, *Hirundo rustica*, and found that secondary sexual characters exhibited significantly greater asymmetry as compared to non-sexual traits. Previous studies had also demonstrated female preference for males with greater symmetry in the same trait, suggesting that sexual characters could be used to indicate male quality (Møller, 1993). Despite the study of fluctuating asymmetry since the late 1950's, debate is still ongoing as to the generality and efficacy of FA as an indicator of environmental stress and fitness. Comparing studies of FA is challenging owing to the vastly different methods by which authors calculate asymmetry, and a number of confounding factors such as measurement error (ME) and the presence of other types of asymmetry described in Sect. 3.1.1. A comprehensive list of statistical and study design prerequisites for any robust and repeatable study of FA is described in Palmer (1994) and Palmer & Strobeck (2003).

3.1.3 Fluctuating Asymmetry in Relation to Radiation

A number of studies have measured fluctuating asymmetry in relation to environmental radiation, with the majority focusing on Chernobyl. To the author's knowledge, no study has assessed FA in non-human organisms at Fukushima. At Chernobyl, a range of organisms including plants (Møller, 1998) birds (Møller, 1993) insects (Møller, 2002) and rodents (Oleksyk et al., 2004) have been monitored, with all studies reporting an increase in FA in populations inhabiting areas of greater contamination. Oleksyk et al., (2004) studied fluctuating asymmetry in the skull of the white necked mouse, *Apodemus flavicollis,* from ten sampling locations across the Ukraine using a morphometric approach. Populations of *A. flavicollis* within the highly contaminated 10km exclusion zone exhibited a high degree of FA, and significant correlations were found between absolute R-L differences and levels of intramuscular ^{137}Cs as measured using a gamma counter. A highly significant negative correlation between distance from the Chernobyl reactor and FA in skull shape was also recorded. The authors proposed a dose threshold of between 0.132 and 0.297 µGy/hr above which levels of FA may increase above background level. Similarly, Møller (1998) studied levels of asymmetry in three plant species (*Robinia pseudoacacia*, *Sorbus aucuparia* and *Matricaria perforata*) along a gradient of radionuclide contamination at Chernobyl. Plants sampled closer to the exclusion zone exhibited levels of FA 3-4 times greater than control regions 225 km away from the zone. Furthermore, developmental instability was found to be positively correlated to levels of ^{137}Cs in soil from the region.

In aquatic systems, studies of FA are more limited. To the author's knowledge, there are only two published studies of FA in Chernobyl impacted lakes. Yavnyuk et al., (2009) studied fluctuating asymmetry in two aquatic species, the floating pondweed *Potamogeton natans* and the zebra mussel *Dreissena polymorpha* from the highly contaminated Glubokoye lake and the Chernobyl cooling pond respectively. In both species an increase in FA was recorded compared to reference sites in other areas of the Ukraine. Lajus et al., (2014) studied fluctuating asymmetry in two cyprinid fish species, roach (*Rutilus rutilus*) and bleak (*Alburnus alburnus*) from the Chernobyl-impacted Bryansk area of Russia. A minor increase in FA was recorded in *A. alburnus* from contaminated areas, however no increase in FA was recorded in *R. rutilus*. The authors attributed the lack of increase in developmental instability to the lower levels of contamination in the sampling locations as compared to other studies.

One of the key limitations of the previous studies in aquatic organisms is a limited number of sample sites and consequently range of dose rates. For example, Lajus et al., (2014) studied only a single contaminated and reference site, which presents difficulties in drawing conclusions regarding the relationship between fluctuating asymmetry and radiation exposure. To address these limitations, the present study sampled six and four localities along a gradient of radionuclide contamination at Chernobyl and Fukushima respectively.

3.1.4 Asellus aquaticus and Eriocheir japonica as Model Organisms

Asellus aquaticus is a detrivorous freshwater isopod commonly found in freshwater systems across Europe. *A. aquaticus* often occupies an overlapping ecological niche to *G. pulex* (see Chapter 2) and plays a similarly fundamental role in leaf litter degradation and nutrient cycling (Graça et al., 1994). *A. aquaticus* is commonly used in ecotoxicology studies of sediment-borne contaminants (De Lange et al., 2005; McCahon & Pascoe, 1988) and has been used as an indicator of water quality (Whitehurst, 1991). More recent studies have focused on population genetics and transcriptomics of *A. aquaticus* (Stahl et al., 2015; Verovnik et al., 2005), emphasising its potential as a model organism. Previous studies have demonstrated the efficacy of FA as indicator of stress in *A. aquaticus*. Savage & Hogarth (1999) exposed *A. aquaticus* to extreme temperatures and demonstrated a positive relationship between temperature stress and FA. A previous study of macroinvertebrate abundance and diversity at eight lakes in the Chernobyl area confirmed the presence of *A. aquaticus* in the region, thus validating the use of *A. aquaticus* as a model in the present study (Murphy et al., 2011).

The Japanese mitten crab, *Eriocheir japonica*, is a grapsid crab distributed in rivers and shallow coastal areas across Japan, Korea and areas of Taiwan (Kobayashi, 2002). *E. japonica* is omnivorous, feeding on both vascular plant detritus and macroinvertebrates (Kobayashi, 2009). This species is catadromous, migrating downstream from rivers to the sea for mating and oviposition (Kobayashi & Matsuura, 1995a). *E. japonica* is euryhaline with a strong capacity to regulate both intra - and extracellular osmolality (Abe et al.,1999). The euryhaline nature of *E. japonica* means that it is of particular interest as a model organism in radioecology, owing to the strong influence of osmoregulation on the uptake and accumulation of radioactive caesium (Arai, 2014). *E. japonica* is a commercially important species for Japanese inland fisheries, with many prefectures implementing stock management and release of juveniles to boost populations (Kobayashi & Matsuura, 1995b). Though the general ecology of *E. japonica* in relation to aquaculture has been well-studied, no studies have monitored the effects of pollution on *E. japonica*. Given the importance of crabs as model organisms for environmental radioprotection (see Sect 1.1.3), this study will provide novel data of the effects of Fukushima-derived radiation on an important species.

3.1.5 Aims & Hypotheses

The aims of the present study were:

a) To assess the impact of chronic low-dose radiation exposure on the developmental stability of natural populations of *Asellus aquaticus* and *Eriocheir japonica*

b) To compare potential differences in the relationship between fluctuating asymmetry and radiation dose rate over time in *Asellus aquaticus*

Owing to the number of studies demonstrating positive relationships between environmental radiation and FA, it was hypothesised that:

- Populations of *A. aquaticus* and *E. japonica* at more contaminated areas of Chernobyl and Fukushima would display elevated levels of FA.

To address aim b), samples of *A. aquaticus* collected in 2004/05 by colleagues at the Belarusian National Academy of Sciences from areas of varying contamination in the Chernobyl region were used. Owing to higher dose rates and the potential for adaptation of biota over longer time scales, it was further hypothesised that:

- A greater effect of radiation on FA would be observed in *A. aquaticus* samples collected closer to the time of the accident compared to samples collected in 2015.

3.2 Materials & Methods

3.2.1 Sampling Sites & Sample Collection

i) Chernobyl

Six lakes in Belarus and Ukraine were selected along a gradient of radionuclide contamination, ranging from 5 km in distance from the CNPP to 225 km. Lakes were selected based on long-term exposure to a gradient of radiation doses and historical measurements of radioactivity. Three of the lakes were located in Belarus, with one (Svatoye 3) located close to the border with Russia. Two of the three Ukrainian lakes were located within 10 km of the CNPP, with an additional site to the South East of Chernobyl close to Kiev (see Figure 3.2). The lakes range in deposition of radiocaesium (in 1986) from 37000 kBq m^{-2} to 10 kBq m^{-2} (see Figure 3.3). Other radionuclides (^{90}Sr and transuranium elements, TUE) also make a contribution to dose. Environmental characteristics were measured using a HANNA 9828 multiparameter probe at three different sub-sites of each lake (HANNA, UK). General environmental characteristics and water quality parameters of the lakes are shown in Table 3.1 All lakes had similar fish communities comprised mainly of perch, roach and rudd (Smith et al., 2005; Murphy et al., 2011). In terms of water chemistry, study lakes were broadly similar with the exception of Yanovsky Crawl, which displayed elevated concentrations of nitrates compared to all other lakes (Data not shown). Previous measurements of calcium concentrations, an important determinant of strontium uptake in aquatic biota (e.g. Chowdhury et al., 2000), in these lakes in 2003 (Cailes, 2006) suggested high concentrations at Glubokoye (30.1 mg/l) relative to all other lakes excluding Dvorische (28.3 mg/l).

Samples of *A. aquaticus* were collected in littoral zones and amongst vegetation in June 2015 at three different sub sites of each lake by kick netting using a 1 mm mesh size net (EFE, UK). Following sieving, *A. aquaticus* were sorted lakeside and immediately preserved in 96% ethanol. Prior to analysis, samples were placed in randomly coded boxes to prevent measurement bias, a pervasive problem in FA studies (Palmer, 1994).

The location and available environmental data for the additional samples collected in 2004/2005 are shown in Figure 3.4 and Table 3.2 respectively. These samples were used to assess potential changes in FA in relation to dose rate over time. Orekhovskiy channel and the Rita river site are located in the Brest region of South-Western Belarus, close to the border with Poland. This area avoided heavy contamination as a consequence of the Chernobyl accident (UNSCEAR, 2000). Consequently, Orekhovskiy channel and the Rita river are considered as reference sites for the samples collected in 2004. Perstok is located in the Belarusian part of the exclusion zone and received high levels of deposition from the Chernobyl accident (see Figure 3.4).

Figure 3.2 – Map of the six study sites sampled in 2015 in relation to the Chernobyl Nuclear Power Plant. Map generated using qGIS (v2.18) with data from DIVA-GIS (Hijmans et al., 2001).

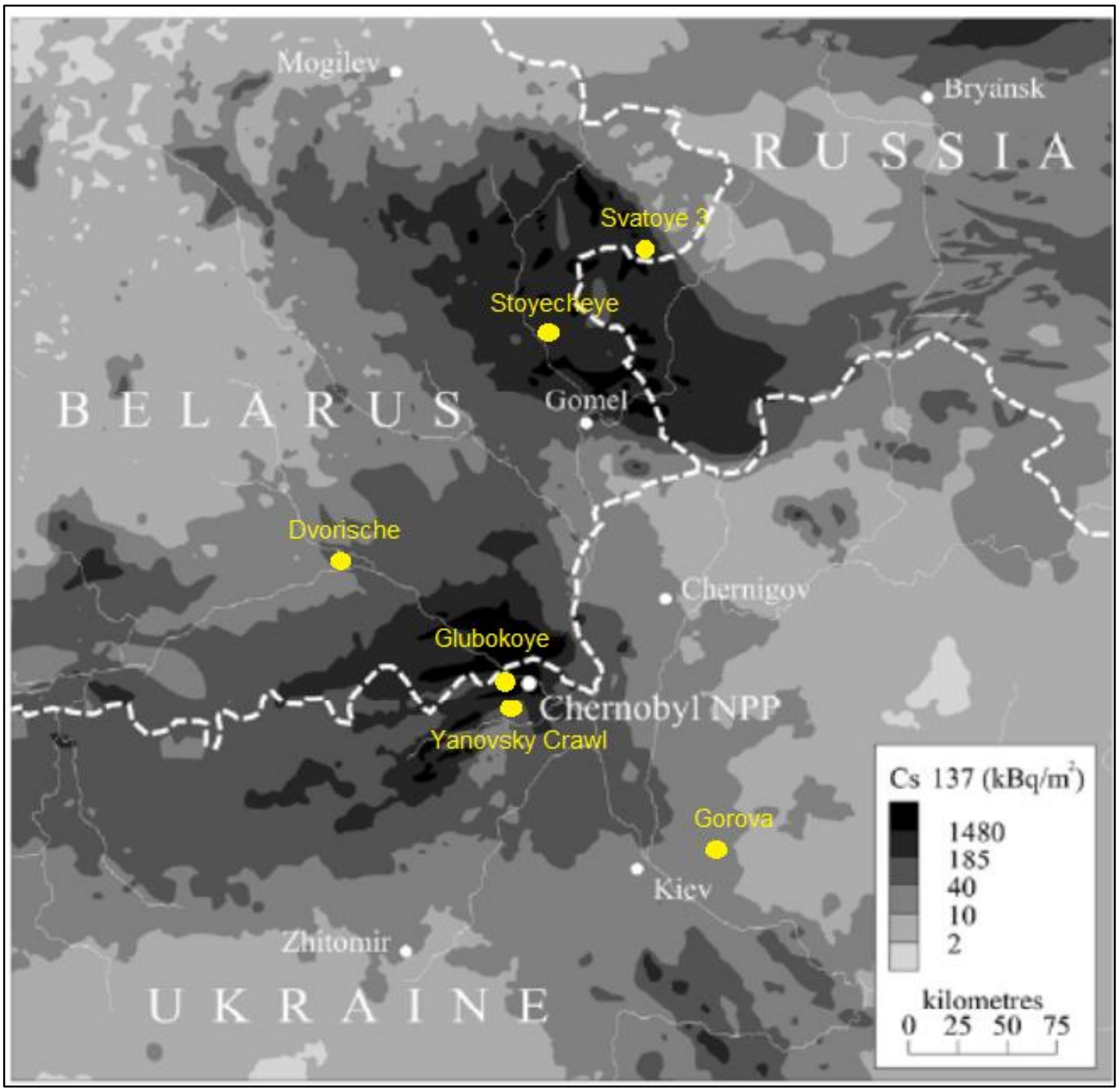


Figure 3.3 - Map of the six sites sampled in 2015 and 2016 with radiocaesium deposition indicated. Modified from Smith & Beresford (2005).

Figure 3.4 – Location of the three additional sites sampled for *Asellus aquaticus* in 2004/2005 in relation to the Chernobyl Nuclear Power Plant.

Table 3.1 – Location and environmental characteristics of the six study lakes at Chernobyl sampled in 2015/2016. ND = No Data. [a] = Data from Smith et al., (2005).

Lake	Latitude	Longitude	Conductivity (µS/cm)	Oxygen Saturation (%)	pH	Temperature (°C)	Max Depth (m)[a]	Surface Area (km^2)[a]	Distance from Chernobyl (km)
Svyatoye-3	53.17	31.86	122.1	81.6	7.8	23.00	2.9	0.250	225
Stoyacheye	52.86	30.91	230	89.4	8.7	24.05	17.0	0.460	157
Dvorsiche	52.01	29.43	200	68.9	7.82	23.73	3.5	0.128	95
Glubokoye	51.44	30.06	184.8	66.3	7.92	24.92	7.3	0.100	10
Yanovsky Crawl	51.41	30.07	275	108.5	9.4	23.29	ND	ND	3
Gorova	50.70	30.70	178.5	185	9.69	22.17	ND	ND	125

Table 3.2 – Sampling date, location and environmental characteristics of sites for collection of *Asellus aquaticus* in 2004/2005.

Site	Sampling Date	Latitude	Longitude	Water Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Total Hardness (mmol/l)	Distance from Chernobyl (km)
Rita River	06.08.05	51.88	24.09	21	7.52	8.75	536	2.43	418
Orekhovskiy Channel	22.08.05	51.97	24.71	19.2	7.50	3	425	2.10	377
Perstok	04.06.04	51.50	30.01	19.3	7.6	11.2	153.5	0.9	14
Svatoye 3	30.08.04	53.17	31.86	16.6	8.3	11.0	123.2	0.7	225
Glubokoye	28.08.04	51.44	30.06	22.1	6.9	6.4	200	1.4	10

ii) Fukushima

Four samples sites were selected ranging from 4 to 44 km in distance from the FDNPP (see Figure 3.5). An overview of the characteristics of each of the sites is shown in Table 3.3. Water quality measurements were taken using a multiparameter probe at three sub-sites of each locality (Horiba U-51, Horiba, Japan). Both ambient and underwater dose rates were assessed at each site using a hand-held gamma spectrometer (TAC T-SP2 Gamma Spectrometer, TAC inc. Japan). For underwater measurements, the spectrometer was housed in waterproof casing and lowered on to the sediment surface where counts were measured for 10 minutes (see Figure 3.6). Sediment samples were taken at four different sub-sites of each location using a Van Veen grab sampler. *E.japonica* samples were collected using cage traps baited with either skipjack tuna or mackerel (see Figure 3.7). Ten traps were set at various areas of each site and were recovered the following day. Collected *E. japonica* individuals were anaesthetised by cooling on ice and returned to the laboratory.

The four sites represented the full range of habitats occupied by *E. japonica*, ranging from the brackish water Matsukawa-ura bay (UR, mean salinity 24.8 ppt) to the freshwater Ukedo river (US) system located in the mountainous area of the exclusion zone. Funazawa (FZ) is a small irrigation pond located within the highly affected Okuma town close to the FDNPP. The Niida river (NR) flows through highly contaminated mountainous areas to lesser contaminated zones within its lower reaches, displaying heterogeneous radiocaesium contamination (Nagao et al., 2015).

Table 3.3 – Characteristics of the four study sites at Fukushima**.** [a] = data from Abe et al., (2017). [b] = Data from Wakiyama et al., (2017). ND = No Data. Ambient and underwater dose rates are displayed as mean ± SD of measurements taken at three sub-sites of each location using a handheld gamma spectrometer.

Site	Latitude	Longitude	Temperature (°C)	pH	Total Dissolved Solids (mg/L)	Salinity (ppt)	Distance from FDNPP (km)	Max Depth (m)	Ambient Dose Rate (µSv/hr)	Underwater Dose Rate (µSv/hr)
Matsukawa-ura (UR)	37.81	140.97	26.60	8.12	23.8	24.8	44.2	4.0[a]	0.06 ± 0.006	0.06 ± 0.007
Funazawa (FZ)	37.40	140.99	26.83	7.56	0.10	0.1	4.3	2.5[b]	1.89 ± 0.20	8.95 ± 0.11
Niida River (NR)	37.64	141.02	26.80	7.98	0.99	0.8	24.4	ND	0.18 ± 0.01	0.09 ± 0.009
Ukedo River (US)	37.51	140.88	21.77	8.5	0.04	0	16.3	ND	7.38 ± 0.61	1.61 ± 0.21

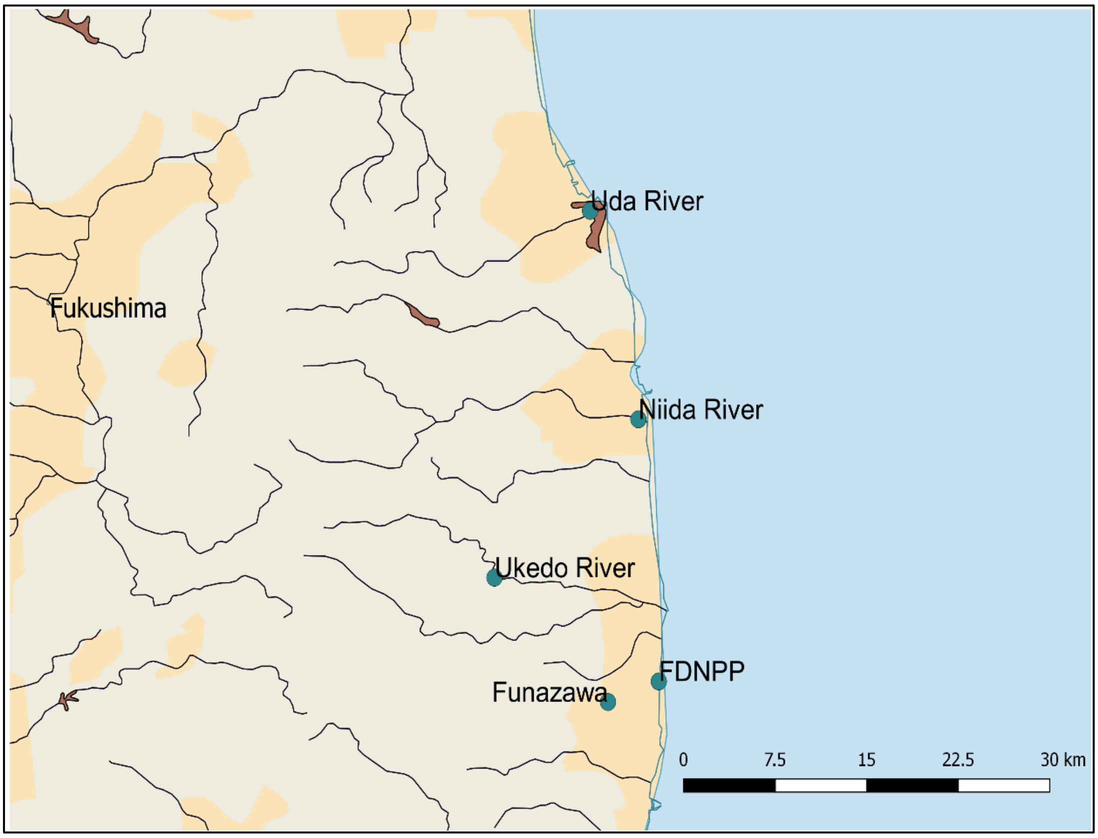


Figure 3.5 – Map of the four study sites at Fukushima with the FDNPP indicated. Generated using qGIS. Map data was obtained from Global Map Japan Version 2 of the Geospatial Information Authority of Japan (Geospatial Information Authority of Japan, 2011).

Figure 3.6 – Measurement of underwater dose rate at Niida River.

Figure 3.7 – Baiting and collection of *Eriocheir japonica* individuals at Matsukawa-ura.

3.2.2 Calculation of Dose Rates

i) Chernobyl

External dose rates were calculated using deposition values of radiocaesium and strontium at sampling sites (see Table 3.4) and dose conversion coefficients (DCC's) based on user inputted geometry data for *A. aquaticus* (height = 2.2mm, width = 1.7mm, length = 4.7mm and mass = 4.1mg) using the ERICA tool (v1.2). DCC's for external dose rates were calculated to be 3.85 x 10^{-4} and 4.91 x 10^{-4} µGy/hr per Bq/kg for ^{137}Cs and ^{90}Sr respectively. Decay corrected activity concentrations of ^{137}Cs and ^{90}Sr in sediments were first calculated (Bq per kg fresh weight) as:

$$C_{sediment} = \frac{(D_{total})\ x\ (e^{-\lambda_r t})}{P_{sediment}\ x\ d_{sediment}}$$

Where

$C_{sediment}$ = Fresh weight activity concentration of sediment Bq kg^{-1}

D_{total} = Total deposition of radionuclide in Bq m^{-2}

λ_r = Decay constant for radionuclide in d^{-1}

t = time in days

$P_{sediment}$ = wet sediment bulk density in kg m^{-3}

$D_{sediment}$ = Depth of sediment in m within which the radionuclide has become mixed

The dose estimates were based on a wet sediment bulk density of 1300 kg m^{-3} and assuming mixing to a depth of 0.15m (Smith et al., 2005). The organism was assumed to occupy the sediment-water interface. The shorter-lived caesium isotope, Caesium-134, (half-life of 2.06 years) was not considered for Chernobyl dose assessments owing to insignificant activity concentrations 30 years post-accident. Sr-90 is associated with fuel particles and shows a rapid decline with distance from Chernobyl relative to radiocaesium (Mueck et al., 2002). Consequently, total doses from Strontium were only considered at Yanovsky Crawl and Glubokoye Lake (3 and 10 km from the Chernobyl NPP respectively) as concentrations at other water bodies are known to be insignificant (Murphy et al., 2011) in comparison with radiocaesium. A generic value of 0.06 µGy/hr was added to estimate cumulative external radiation doses in study lakes to account for natural background radiation (Murphy et al., 2011). Following the decay of ^{137}Cs and ^{90}Sr over long time scales, the actinides Americium and Plutonium will become the major contributors to dose at Chernobyl (Smith & Beresford, 2005). Measurement of activity concentrations of these radionuclides, along with ^{137}Cs and ^{90}Sr in a bulk sample of *A. aquaticus* from each lake is ongoing with collaborators at the University of Southampton to validate the dose estimates from ERICA.

Table 3.4 - Estimated activity concentrations of sediment and concomitant external doses received by *Asellus aquaticus* based on radiocaesium and strontium deposition at six localities in Belarus and Ukraine. Data from Smith et al., (2005) unless otherwise stated. [a] data from Sansone & Voitsekhovitch (1996), [b] data from Nazarov & Gudkov (2009) [c] data from Cort (1998). [d] Data from Ukrainian Hydrometerological Institute, Kiev.

Lake	^{137}Cs deposition at site (kBq m^{-2})	^{137}Cs activity of sediment (Bq kg^{-1})	External Dose Rate ^{137}Cs (µGy/hr)	^{90}Sr Deposition at Site (kBq m^{-2})	^{90}Sr Activity of Sediment (Bq kg^{-1})	External Dose Rate ^{90}Sr (µGy/hr)	Total External Dose Rate (µGy/hr)
Glubokoye 2015	15500[a]	40706	7.84	12000	30526	7.51	15.35
2004		52406	10.1		39776	13.7	23.9
Yanovsky Crawl	14800[b]	38867	7.5	16300	41465[a]	10.2	17.7
Perstok (2004)	3700	12513	2.41	22	96.9	0.002	2.47
Svyatoye 3 2015	1748	4591	0.886	ND	N/A	N/A	0.886
2004		5912	1.14	ND	N/A	N/A	1.14
Stoyacheye	288	756	0.145	ND	N/A	N/A	0.145
Dvorische	100[c]	262	0.0513	ND	N/A	N/A	0.0513
Orekhovskiy Channel (2005)	10	33.04	0.0122	ND	N/A	N/A	0.072
Rita River (2005)	10	33.04	0.0122	ND	N/A	N/A	0.072
Gorova	10	26	1.93E-03	ND	N/A	N/A	1.93E-03

Internal dose rates were calculated based on average measurements of ^{137}Cs and ^{90}Sr in lakes at various depths in 2003 (see Table 3.5). Dose estimates were made using the ERICA tool (V 1.2) and DCC values of 1.9 x 10^{-4} and 6.3 x 10^{-4} µGy/hr per Bq/kg were calculated for ^{137}Cs and ^{90}Sr respectively. Since no data exists for activity concentrations of ^{137}Cs and ^{90}Sr in the less contaminated lakes, a value of 0.063 µGy/hr was added to account for background exposure following Kryshev & Sazykina (1995). Internal and total dose rates are shown in Table 3.5.

Table 3.5 - Estimated internal and total doses received by *Asellus aquaticus* at nine lakes based on activity concentrations of ^{137}Cs and ^{90}Sr in water and sediment. ^{137}Cs and ^{90}Sr in water based on average measurements at different depths in 2003.[a] Data based on average measurements from 1997-2008 from Nazarov and Gudkov (2009).

Lake	^{137}Cs in water (Bq l^{-1})	Internal Dose ^{137}Cs (µGy/hr)	^{90}Sr in water (Bq l^{-1})	Internal Dose ^{90}Sr (µGy/hr)	Total Internal Dose (µGy/hr)	Total Dose Rate (µGy/hr)
Glubokoye 2015	7.57[d]	1.16	77.7	7.6	11.66	27.1
2004	9.77	1.49	101.53	9.8	15.19	35.2
Yanovsky Crawl	2.2	0.336	18.7[a]	2.53	2.866	20.6
Perstok	13.1	2.00	21.5	2.91	4.91	7.91
Svatoye #3 2015	7.8	1.19	N/A	N/A	1.19	2.2
2004	10.07	1.54	N/A	N/A	N/A	2.74
Stoyacheye	4.24	0.647	N/A	N/A	0.647	0.872
Dvorische	4.29	0.655	N/A	N/A	0.655	0.786
Orekhovskiy Channel (2005)	ND	0.063*	N/A	N/A	N/A	0.069
Rita River (2005)	ND	0.063*	N/A	N/A	N/A	0.069
Gorova	ND	0.063*	N/A	N/A	0.063	0.064

ii) Fukushima

For accurate dose estimates, activity concentrations in sediment were measured using a high-purity germanium detector (Canberra GC4020, Canberra, USA) at the Institute of Environmental Radioactivity (IER), Fukushima University. Three sediment samples per site were first dried for two days at 70°C using a Yamamoto DY600 Drying Oven, then homogenized thoroughly using a pestle and mortar. Samples were added to a pre-weighed 100ml U8 Yamayu Puretube (Yamayu, Japan) and the exact weight and height determined using a Sartorius Secura 224-1S balance (Sartorius, Germany, Precision ± 0.1mg) and digital calipers (TESA Digit-Cal, TESA, Switzerland, Precision ± 0.01mm) respectively. Samples were counted from 1800 to 54000 seconds depending on the degree of contamination at the corresponding site. Strontium-90 was not considered for dose assessments owing to negligible activity concentrations (< 30 Bq kg^{-1}) measured within areas impacted by the nuclear accident (Sahoo et al., 2016). Though the Fukushima accident caused a slight increase in ^{90}Sr (Ministry of Education, Culture, Sports, Science and Technology Japan [MEXT], 2012; Sahoo et al., 2016), activity concentrations are not considered to be radiologically significant in terms of doses to biota. Activity concentrations in sediment and water were inputted in to the ERICA tool to calculate dose rates for *E. japonica*. Doses were based on the ERICA tools reference crustacean which was assumed to occupy the sediment surface. User inputted geometry was not used for *E. japonica* dose assessments, since the ERICA reference crustacean is based on a crab. Activity concentrations of sediment and water are shown in Table 3.6 Table 3.7 shows the calculated internal, external and total dose rates received by *E. japonica* using the ERICA tool.

Table 3.6 – Measured activity concentrations of radiocaesium (^{134}Cs + ^{137}Cs) in sediment and water at four sites of varying contamination at Fukushima. Sediment activity concentrations are shown as ± counting error. [a] = Data decay corrected to sampling date from Wakiyama et al., (2017). [b] = Data decay corrected to sampling date from Ochiai et al., (2015).

Site	^{137}Cs Activity Concentration in Sediment (Bq kg^{-1})	^{137}Cs Activity Concentration in Water (Bq l^{-1})	^{134}Cs Activity Concentration in Sediment (Bq kg^{-1})	^{134}Cs Activity Concentration in Water (Bq l^{-1})
Matsukawa-ura (UR)	63.85 ± 1.6	ND	8.16 ± 0.53	ND
Funazawa (FZ)	154398 ± 398	13[a]	19286 ± 333	ND
Niida River (NR)	497.25 ± 4.4	0.24[b]	62.1 ± 1.10	0.154[b]
Ukedo River (US)	17196 ± 86.86	0.10[b]	2152 ± 26.3	0.048[b]

Table 3.7 – Calculated dose rates received by the Japanese mitten crab, *Eriocheir japonica*, at four sites of varying contamination at Fukushima. Dose rates were calculated based on activity concentrations of ^{134}Cs and ^{137}Cs in sediment and water using the ERICA tool.

Site	^{134}Cs External Dose Rate (µGy/hr)	^{134}Cs Internal Dose Rate (µGy/hr)	^{137}Cs External Dose Rate (µGy/hr)	^{137}Cs Internal Dose Rate (µGy/hr)	Total Dose Rate (µGy/hr)
Matsukawa-ura (UR)	0.004	0.0001	0.011	0.0007	0.016
Funazawa (FZ)	8.88	0.10	28.6	0.553	38.133
Niida River (NR)	0.029	0.020	0.092	0.0418	0.18
Ukedo River (US)	0.990	0.0063	3.180	0.017	4.19

3.2.3 Calculation of Fluctuating Asymmetry

i) Asellus aquaticus

Four traits were selected for analysis of FA in *A. aquaticus*; first antenna length, propodos and merus width of the first paraeopod and carpus width of the second paraeopod. Selection of these traits was based on previous studies using similar characters to demonstrate a relationship between environmental stress and FA in *Asellus aquaticus* (e.g. Savage & Hogarth, 1999). A preliminary study (data not shown) further demonstrated that the selected traits were robust, easily quantifiable and damage during handling would not bias measurements. As an additional measure, the number of segments of the first antenna was quantified following three replicate counts. Asymmetry in segment number has been previously been demonstrated in *A. aquaticus* and appears to be linked to antennal length asymmetry (Savage & Hogarth, 1999).

A list of the numbers of individuals analysed per site is shown in Table 3.8. Organisms were first sexed by analysis of the pleopods and measurements of body length conducted following Bertin et al., (2002). Characteristics were dissected, mounted on slides and photographed using a Leica DFC310 camera following flattening under a 22 x 40 mm coverslip. Two independent blind measurements were taken on each trait using ImageJ (v 1.48). All measurements were conducted by one researcher (NF) over a two week period to minimise inter-observer variability (Lee, 1990). FA was calculated using the FA1 index as: FA = Mean $|R-L|$, where R and L represent the right and left side trait measurements in µm respectively**.** For comparisons of mean pooled FA values across sites, the size-corrected FA2 index was used: FA2 = mean $[|R-L|/(R+L)/2)]$ to account for differences in trait size as suggested in Palmer (1994). Finally, composite FA (CFA) values were calculated using the CFA2 index (Leung et al., 2000):

$$CFA2 = \sum[|FAij| / |FAj] / Nt$$

Where FAij is the deviation from symmetry of a trait j in an individual i, and FAj is the average absolute deviation from symmetry of a given trait j for the entire sample. Composite FA measures provide a host of advantages as compared to conventional analyses using single traits, including an increased probability of detecting differences in FA between populations and FA-stress relationships (Leung et al., 2000).

Table 3.8. – Number of individuals analysed for fluctuating asymmetry per site for the Chernobyl study.

Site	No. of *A. aquaticus* Individuals Analysed
Gorova	71
Orekhovskiy Channel	50
Rita River	42
Dvorische	66
Stoyecheye	63
Svatoye 3 2015	60
2004	26
Perstok	20
Yanovsky Crawl	70
Glubokoye 2015	71
2004	53

ii) *Eriocheir japonica*

In *E. japonica,* eight traits were measured; the merus length and width of the fourth and first paraeopods, carpus length and width of the third paraeopod and propodos length and width of the second paraeopod. These traits were based on previous studies of fluctuating asymmetry in crabs (e.g. Duarte et al., 2008; Li, 2002). Individuals were first sexed by examination of the abdomen and a range of basic morphometric data was collected including carapace width, carapace length, eye cavity length and abdominal length (Duarte et al., 2008). Measurements were taken twice over a two-week period using digital calipers (TESA Digit-Cal, Precision ± 0.01mm, TESA, Switzerland). A total of 3168 measurements on 99 individual crabs were conducted. The specific numbers of individuals per site used for FA analyses is shown in Table 3.9. The FA indices FA1, FA2 and CFA2 were calculated as described in the previous section.

Table 3.9 – Numbers of *E. japonica* analysed for fluctuating asymmetry per site.

Site	No. of *E. japonica* Analysed
Matsukawa-ura	31
Niida River	17
Ukedo River	24
Funazawa	27

3.2.4 Statistical Analyses

For all data sets, the significance of all between-sides variation relative to measurement error (ME) was first assessed using a two-way mixed model ANOVA design as described by Palmer (1994). ME is often of similar magnitude to FA and may lead to spurious FA x stress relationships, therefore the significance of FA to ME is fundamental to any study of fluctuating asymmetry (Palmer & Strobeck, 2003). The model was applied to each lake x trait combination with individual as a random factor and orientation as a fixed factor. This method also tests for the presence of directional asymmetry (see Sect 3.1) in measured morphometric data. As an additional measure, one-sample t tests were performed against a mean of zero to further test for the presence of DA (Baker & Hoelzel, 2013). These procedures cannot deduce whether the observed pattern of variation is due to true FA or antisymmetry, a pattern of variation characterised by a bimodal distribution either side of zero (see Figure 3.1c). Traits displaying antisymmetry are typically not considered useful indicators of developmental stability, since they may have a genetic basis (Palmer &

Strobeck, 1992). Therefore, the presence of asymmetry must be tested for in studies of FA and traits shown to exhibit significant departures from normality are typically excluded from subsequent analyses (Palmer & Strobeck, 1994). Tests for asymmetry were performed using conventional kurtosis statistics and one-sample Kolmogorov-Smirnov tests. Finally the presence of size dependence, the association between organism size and magnitude of FA, must be accounted for as it can confound observed differences in FA between sites if different size ranges are present. Non-parametric tests of association are preferred for such analysis since they do no assume homogeneity of variance (Palmer & Strobeck 2003). Spearman's rank-order correlation coefficients were therefore used to test the degree of size dependence of each trait with body length in *A. aquaticus* and carapace width in *E. japonica*.

Differences in overall pooled asymmetry between the two sampling years in *A. aquaticus* was first analysed using a GLM. In order to test for differences in FA between sampling sites in *A. aquaticus* and *E. japonica,* a GLM was used wherein sampling site and sex were fixed factors and trait a random factor to ascertain the influence of sex on observed FA values. Size corrected FA values (index FA2) averaged across replicate measurements were used for these models (Arnqvist & Mårtensson, 1998). Owing to the non-normal distribution of the metrical segment asymmetry data in *A. aquaticus*, a Kruskal-Wallis H test was used to test for differences between sample sites for this trait. Post-hoc Dunn's tests were applied with the Benjamini-Hochberg correction via the dunn.test package (Dinno, 2015).

The relationship between FA and radiation dose rates were tested using multiple approaches. Where no significant differences between traits existed, pooled size-corrected FA2 values were used for testing the relationship between dose rate and levels of FA (Lajus et al., 2015). Where levels of FA differed significantly between traits, individual trait x FA relationships were assessed using Spearmans rank-order correlation (Michaelsen et al., 2015). Finally, the composite FA index was used to test FA and dose relationships owing to the increased statistical power associated with this index (see Leung et al., 2000 for further discussion). Relationships between calculated dose rates and log transformed environmental parameters with FA were tested using Spearmans rank-order correlation or linear regression. Statistical analyses were performed using either IBM SPSS (v 22.0) or R Studio (v1.0136).

3.3 Results

3.3.1 Estimated Radiation Dose Rates Received by Biota

i) Asellus aquaticus

Tables 3.4 and 3.5 display the estimated external and internal radiation doses at each of the sampling sites based on ^{137}Cs and ^{90}Sr activities in sediment and water. Maximum and minimum total doses were calculated at Glubokoye lake in 2004 (35.1 µGy/hr) and Gorova respectively (0.06 µGy/hr, See Table 3). Dose rate estimates based on deposition of radionuclides at study sites are subject to a significant degree of uncertainty, owing to the necessary simplifications and assumptions made to calculate these values (see Murphy et al., 2011). However, direct measurements of external radiation doses at Svyatoye-3 by Pungkun (2012) using a thermoluminescent dosimeter (TLD) array provided good agreement with dose estimates in the present study (0.8 – 1.8 µGy/hr for measured average sediment concentrations), suggesting estimates in the present study provide an adequate assessment of radiation doses received by biota.

ii) *Eriocheir japonica*

Total dose rates from ^{134}Cs and ^{137}Cs to *E. japonica* are shown in Table 3.7. Maximum and minimum total dose rates were calculated at FZ and UR, being 38.133 and 0.016 µGy/hr respectively. These dose rates are significantly higher than were measured on the sediment surface using a handheld gamma spectrometer. For example, dose rates of 38.133 µGy/hr were calculated using ERICA for *E. japonica* compared with 8.95 µSv/hr measured using the gamma spectrometer. However, the handheld gamma spectrometer was used only to provide a rough indication of the gradient in dose rates between sites and was not calibrated specifically for underwater measurements. Consequently, ERICA calculated dose rates were used for subsequent analysis of the relationship between dose rate and FA.

3.3.2 Departures from Normality and Measurement Error

i) *Asellus aquaticus*

Conventional kurtosis statistics of each trait x lake combination are displayed in Appendix Table A1. The majority of lake x trait combinations demonstrated slight leptokurtosis which is not attributed to antisymmetry. Where platykurtic distributions existed, Kolmogorov-Smirnov tests and visual inspections of FA frequency distributions revealed no significant departures from normality ($p > 0.05$). Analysis of the metrical dataset (number of antennal segments) revealed a leptokurtic distribution (high, narrow peak) which was significantly different from the normal distribution (Kolmogorov – Smirnov test, $p < 0.05$). This was attributed to the low range of observed R-L values in the data and is frequently observed in metrical traits (e.g. pectoral fin rays in fishes [Johnson et al., 2004; Young et al., 2009]). Since antisymmetry is characterised by a platykurtic distribution (Palmer & Strobeck, 1986) and segment asymmetry did not significantly differ from 0, antisymmetry was not considered. The two-way ANOVA procedure described in Sect. 2.3 revealed that the magnitude of between-sides variation was greater than measurement error ($p < 0.001$) in all cases indicating the significance of observed asymmetry relative to ME and repeatability of the traits measured. By excluding a significant effect of ME on patterns of FA variation, this test allows for robust analyses of FA differences between populations.

ii) *Eriocheir japonica*

Kurtosis statistics for *E. japonica* lake x trait combinations are shown in Appendix Table A2. Significant departures from normality were detected in the propodos width of the second paraeopod and the merus length and width of the first paraeopod (Kolmogorov-Smirnov Test, $p < 0.05$), suggesting antisymmetry. Traits displaying antisymmetry are typically not considered useful indicators of developmental stability, since they may have a genetic basis

(Palmer & Strobeck, 1992). These traits were therefore excluded from subsequent statistical analyses. Significant orientation x individual interaction terms for all site x trait combinations validated the presence of FA in relation to ME and the repeatability of the traits chosen in the present study.

3.3.3 Size Dependence

No significant relationship between absolute FA values and *E. japonica* carapace width or *A. aquaticus* body length was observed (Spearmans rho, $p > 0.05$). This suggested that FA was unrelated to the size of individuals and size dependence was not considered in the present study.

3.3.4 Fluctuating Asymmetry

i) *Asellus aquaticus*

Significant differences in levels of pooled size-corrected FA were recorded between 2015 and 2004/2005 samples (GLM, $F_{1, 2292} = 35.037$, $p < 0.05$) therefore data was analysed for each sampling period separately. For the 2015 samples, significant (ANOVA, $F_{5, 1553} = 3.24$, $p < 0.05$) differences in size-corrected FA was observed between sites, indicating different levels of developmental stability between lake populations (see Table 3.10). A post-hoc Bonferroni test revealed significant differences in FA values between Svyatoye-3 and all sites excluding Dvorische and Glubokoye lake (see Figure 3.8) when information from multiple traits was considered ($p = < 0.05$). At Svyatoye-3, mean pooled asymmetry of four traits was 0.063 (n = 228), significantly greater than Yanovsky Crawl (0.043, n = 274), Stoyacheye (0.032, n = 271) and Gorova (0.045, n = 277). First antennae length exhibited the greatest degree of asymmetry overall (0.052, n = 398), however differences between trait were not significant (see Table 3.10, $p > 0.05$). Lowest mean asymmetry values were recorded for the carpus of the second paraeopod (0.044, n = 394). The general linear model revealed no significant differences in asymmetry between sexes, independent of locality or specific trait measured ($p > 0.05$, See Table 3.10).

No significant differences in the magnitude of segment asymmetry (calculated as |R-L|) were recorded between sampling sites (Kruskal-Wallis H, $p > 0.05$). Maximum right-left differences of 4 segments were displayed at lakes Glubokoye and Svyatoye-3. The highest % of individuals exhibiting segment asymmetry was Svyatoye-3, wherein 35.6% of asellids exhibited asymmetry in segment number. The lowest number of individuals displaying segment asymmetry (21.7%) was recorded at Gorova.

Table 3.10 – Results of the GLM with a mixed model ANOVA design performed on size corrected asymmetry data in *A. aquaticus* collected in 2015.

Source of Variation	Mean Square	F Value	*Df*	p value	Interpretation
Site	0.014	3.238	5	0.035	FA varies between localities
Sex	0.000	0.704	1	0.803	FA does not differ between sexes
Trait	0.006	1.513	3	0.396	FA does not vary dependent on trait
Site*Sex	0.001	0.309	5	0.900	FA differences between sexes not dependent on site
Site*Trait	0.004	2.383	15	0.052	FA differences between sites not dependent on trait
Sex*Trait	0.003	1.402	3	0.280	FA difference between traits not dependent on sex

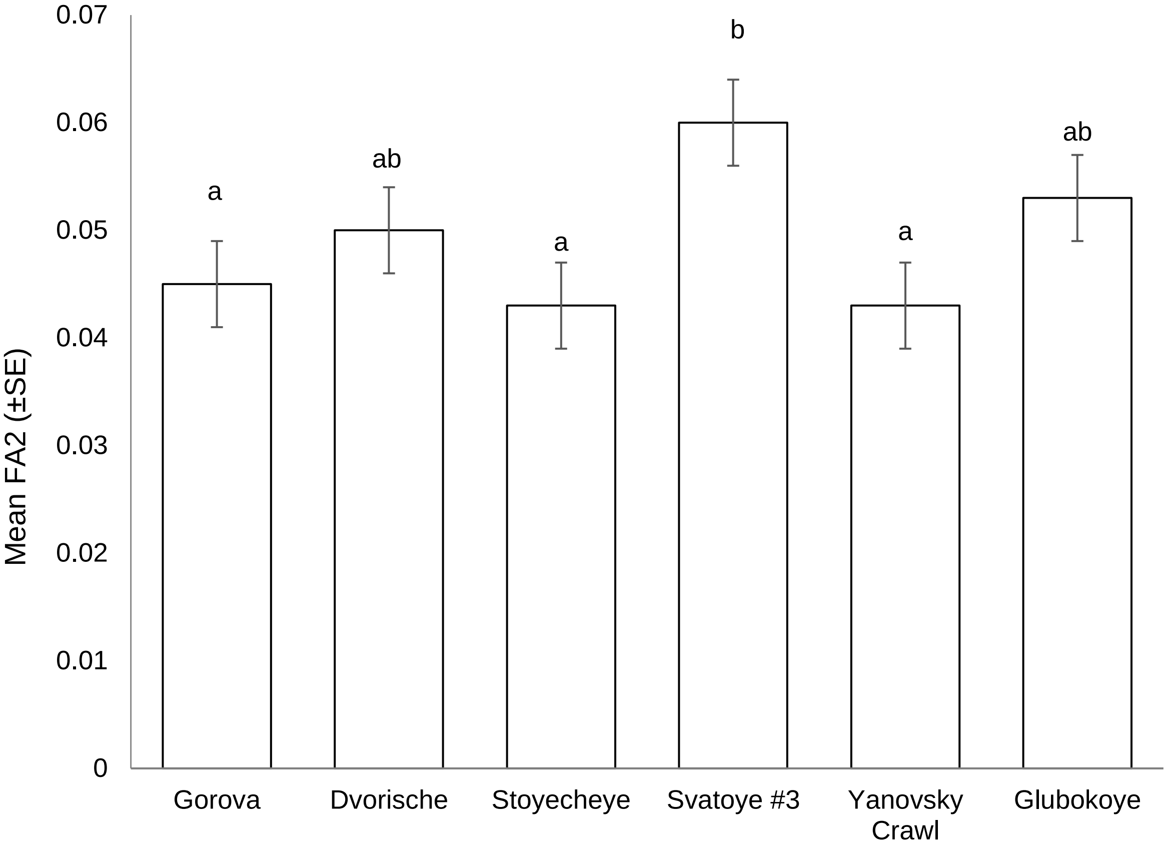


Figure 3.8 – Mean pooled size-corrected fluctuating asymmetry (FA2) in *A. aquaticus* collected from six sites of varying contamination at Chernobyl in 2015. Sites are ordered in increasing contamination from left to right. Matching letters denotes no significant differences ($p > 0.05$), whereas different letters represent significant differences ($p > 0.05$). Error bars are standard error of the mean.

For samples collected in 2004/2005, no significant differences between sample sites was recorded, though this approached significance (see Table 3.11, $F_{4,\ 673} = 2.92$, $p = .067$). However, highly significant differences between traits were recorded ($F_{3,\ 672} = 7.94$, $p < 0.01$) due to significantly greater FA in both the merus and carpus length as compared to the propodos length and first antennal length (see Figure 3.9, Bonferroni post-hoc test, $p < 0.05$). The non-significant interaction term between site and trait ($F_{12,\ 672} = 1.12$, $p > 0.05$) indicated that this was independent of sampling site. Greatest asymmetry was observed at the most contaminated site, Glubokoye (0.032, n = 191) with the lowest overall FA observed at the reference site, Orekhovskiy Channel (0.024, n =196). Given the highly significant differences between traits in the 2004 samples, subsequent analysis of radiation x FA relationships were conducted on a trait-by-trait basis using the FA1 (|R-L|) index (see Figure 3.13).

Significant differences in metrical segment asymmetry were observed between sample sites (Kruskal-Wallis H, $\chi^2 = 15.21$, df = 4, $p < 0.05$). This was owing to significantly greater FA at Glubokoye as compared to all other sites excluding Perstok (Dunn's test, Benjamini-Hochberg correction, $p < 0.05$).

Table 3.11 – Results of the GLM with a mixed model ANOVA design performed on size corrected asymmetry data in *Asellus aquaticus* collected in 2004.

Source of Variation	Mean Square	F Value	*Df*	p value	Interpretation
Site	.025	2.918	4	.067	FA does not vary between localities
Trait	0.068	7.938	3	.002	FA varies depending on trait
Site*Trait	0.009	1.115	12	.344	FA differences between trait not dependent on site

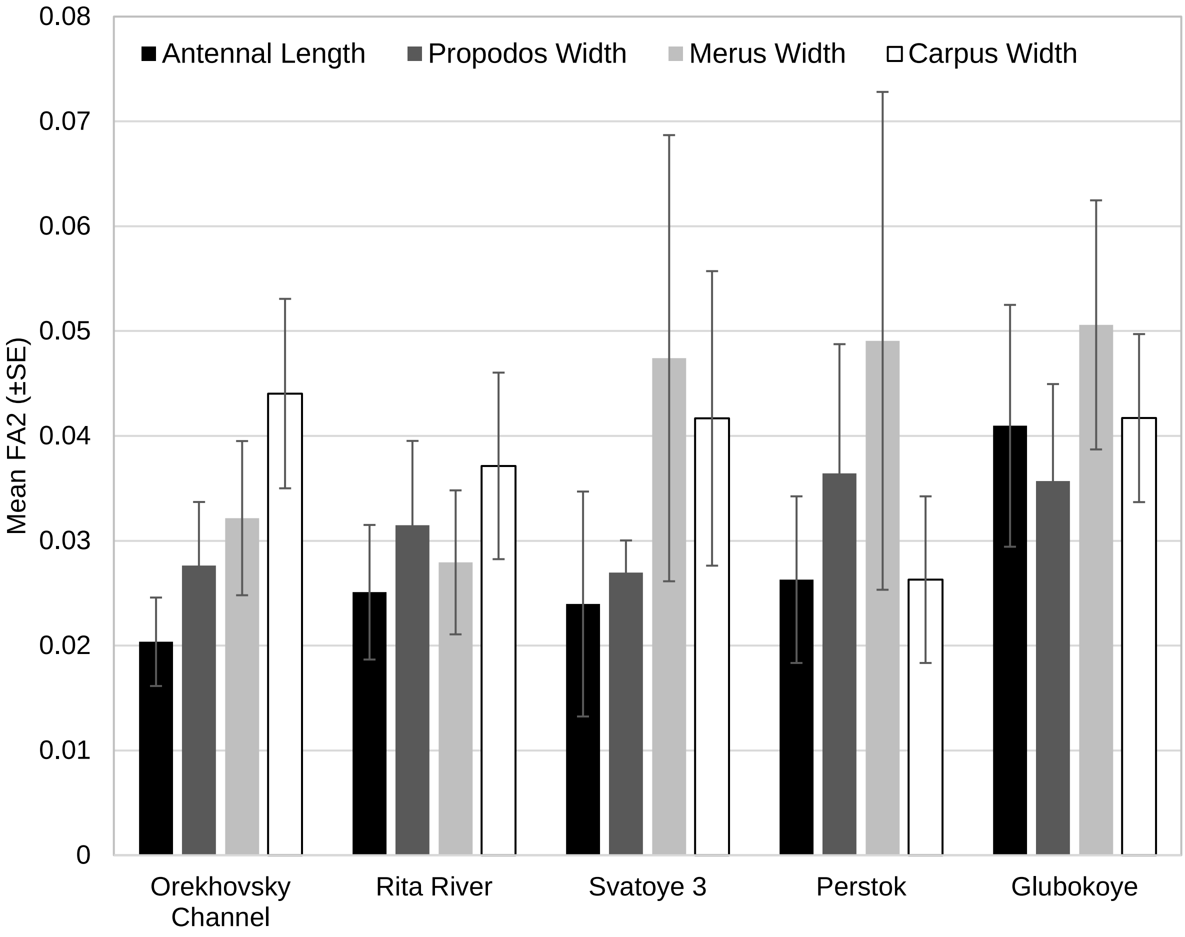


Figure 3.9 – Mean size-corrected fluctuating asymmetry (FA2) in *Asellus aquaticus* collected in 2004/2005 at five sites of varying contamination at Chernobyl. Error bars are ± standard error of the mean.

ii) *Eriocheir japonica*

In *E. japonica,* no significant differences were observed between sample sites ($F_{3, 435} = 3.11$, $p > 0.05$, See Table 3.12) or sexes ($F_{1, 435} = .480$, $p > 0.05$), suggesting that levels of FA did not vary at sites of varying contamination or between sexes. Highly significant differences in the level of FA between traits were recorded ($F_{4, 435} = 29.23$, $p < 0.001$), indicating that levels of developmental stability were dependent on the morphological trait measured. This was owing to significantly lower FA values in the merus length and width of the fourth paraeopod as compared to all other traits (see Figure 3.10, Pairwise comparisons, Bonferroni correction, $p < 0.05$). Greatest pooled values for size-corrected FA were observed at the Niida River site (Mean 0.054, n = 80), with the lowest recorded at the most contaminated location, Funazawa (Mean 0.038, n = 130).

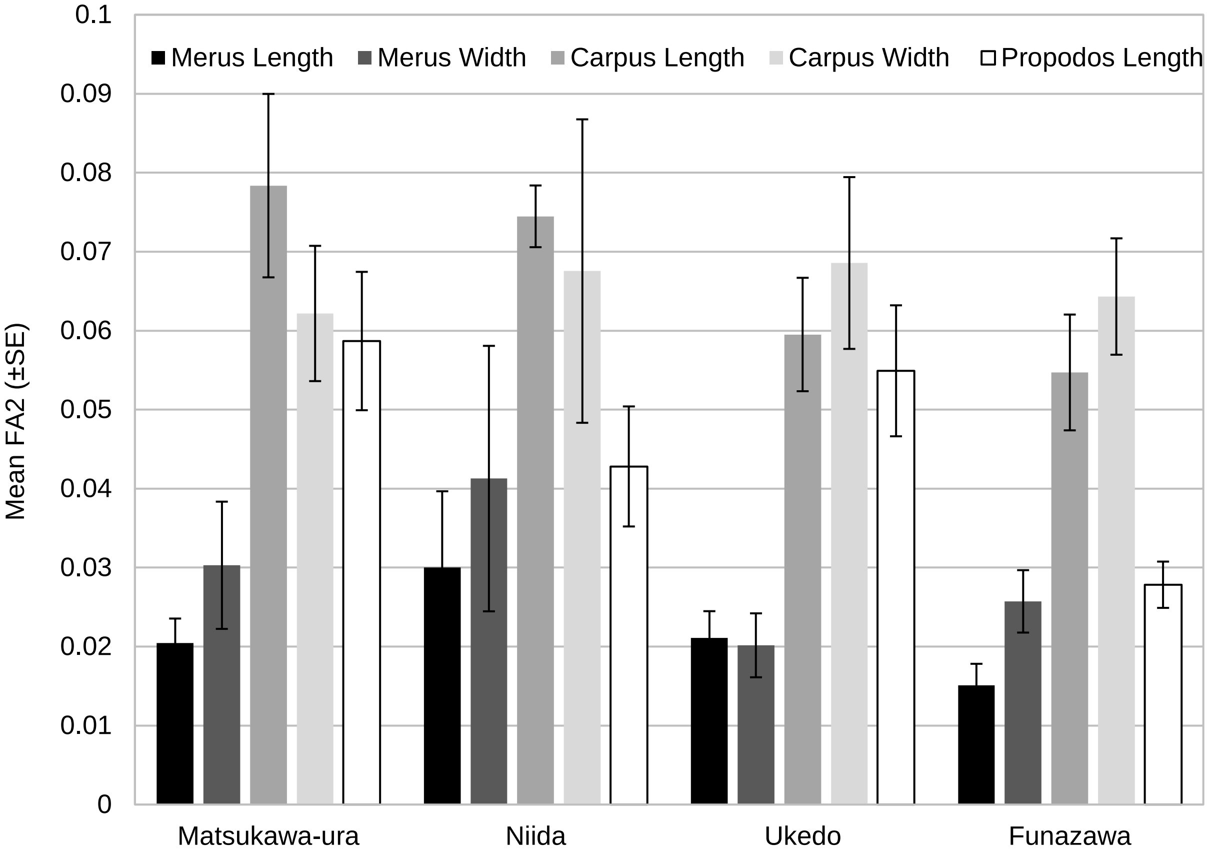


Figure 3.10 – Mean size-corrected fluctuating asymmetry (FA2) in five morphological traits in *Eriocheir japonica* along a gradient of contamination at Fukushima. Sites are in order of increasing contamination from left to right. Error bars are standard error of the mean.

Table 3.12 – Results of the GLM for size-corrected FA data in *E. japonica*

Source of Variation	Mean Square	F Value	*Df*	p value	Interpretation
Site	.026	3.111	3	.067	FA does not vary between localities
Sex	0.003	.480	1	.526	FA does not differ between sexes
Trait	0.277	29.225	4	.000	FA varies depending on trait
Site*Sex	0.017	4.503	3	0.025	FA differences between sexes dependent on site
Site*Trait	0.008	2.130	12	0.052	FA differences between sites not dependent on trait
Sex*Trait	0.005	1.325	4	0.307	FA difference between traits not dependent on sex

3.3.5 Fluctuating Asymmetry in Relation to Radiation & Environmental Parameters

i) Asellus aquaticus

In 2015, mean asymmetry at the most highly contaminated lake, Glubokoye (total dose rate of 27.1 µGy/hr) was 0.053, 1.2 times greater than individuals from the site of lowest contamination, Gorova (total dose rate of 0.06 µGy/hr) although these differences were non-significant (Bonferroni, $p > 0.05$). Mean asymmetry at Yanovsky crawl, with total dose rates of 20.6 µGy/hr, was 0.043, within the range of sites exhibiting dose rates up to two orders of magnitude lower (e.g. Gorova). The relationship between asymmetry and radiation dose rate along the contamination gradient is shown in Fig. 3.11. The value for the regression slope was 0.008 (see Figure 3.11), demonstrating the lack of relationship between the two variables which was non-significant ($p > 0.05$). Furthermore, no significant relationship between CFA2 and dose rate was recorded (Spearmans rho = .006, $p > 0.05$). This suggests that differences in levels of developmental stability in metrical traits was not related to radionuclide contamination in samples collected in 2015.

Antennal segment asymmetry and total dose rate approached a positive relationship (R^2 = 0.51, See Figure 3.12), however this relationship was non-significant (Spearman's rank-order correlation, $p > 0.05$). The raw data showed that in 72% of cases, asymmetry of segment number and antennal length was in the same direction, suggesting that antennal asymmetry in length and number of segments is linked as originally proposed in Savage & Hogarth (1999). No significant relationship between pooled FA and any of the measured environmental parameters including conductivity (R^2 = 0.063, p = 0.330, df = 1, F = 1.015), temperature (R^2 = 0.012, p = 0.663, df = 1, F = 0.198), pH (R^2 = 0.067, p = 0.301, df = 1, F = 1.141) and dissolved oxygen levels (R^2 = 0.017, p = 0.603, df = 1, F = 0.281) was recorded. This suggests that the measured confounding factors did not impact levels of FA.

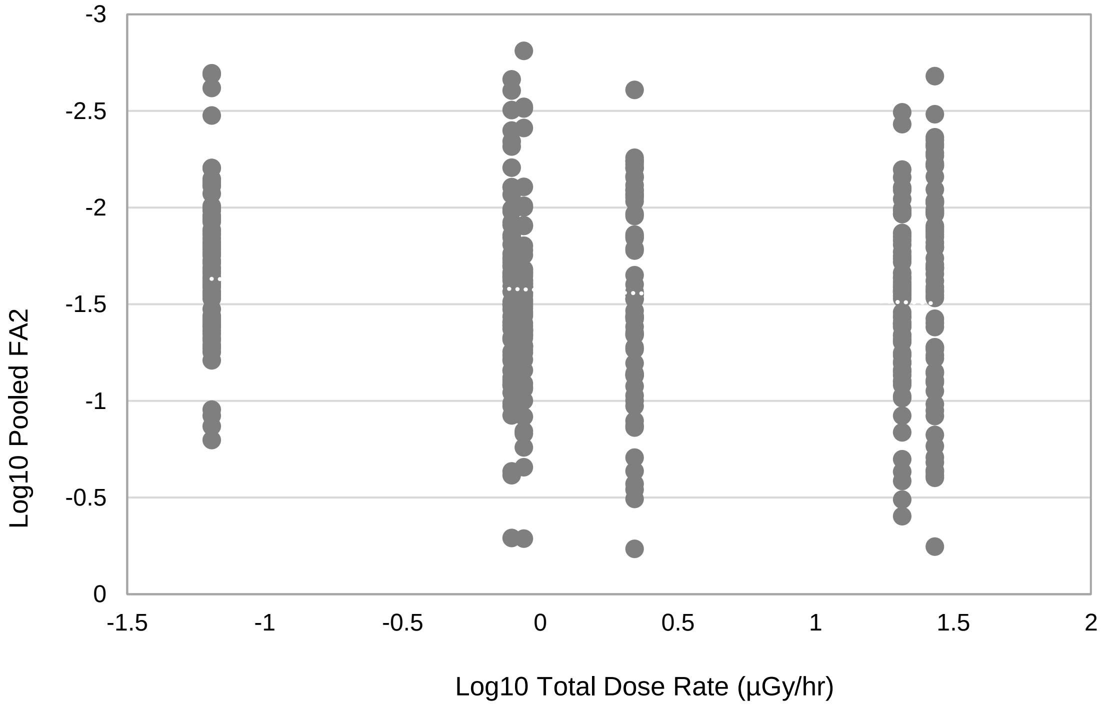


Figure 3.11 – Relationship (R^2 = .008, $p > 0.05$) between size-corrected asymmetry and dose rate along a gradient of radionuclide contamination at Chernobyl in 2015.

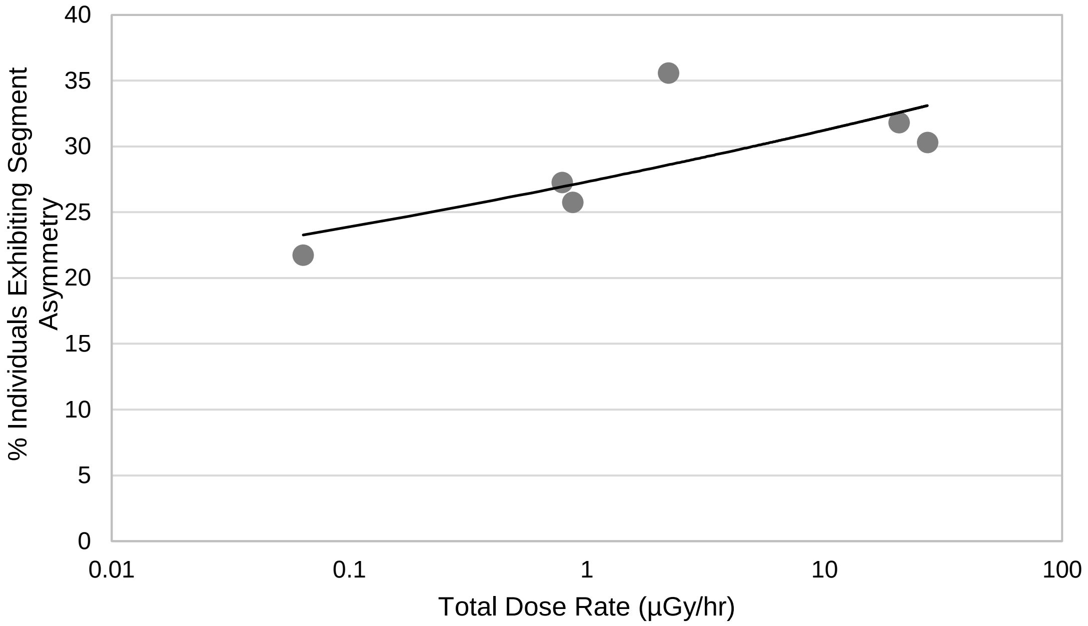


Figure 3.12 – Relationship ($R^2 = 0.51$, $p > 0.05$) between the % individuals exhibiting segment asymmetry and total dose rate at Chernobyl in 2015.

In 2004/2005 samples, significant FA x dose rate relationships were detected for two of the five analysed traits (segment asymmetry and first paraeopod merus width, Spearmans rho, $p < 0.05$, see Figures 3.13C and 3.13E) when using the FA1 index. Figure 3.13 shows the relationship between FA and dose rate for each trait. However, when considering multiple traits using the size-corrected FA2 index, no significant differences between sites of varying contamination were recorded (see Sect 3.4). No significant relationship between levels of CFA and radiation dose rate was recorded (Spearmans rho = - .005, $p > 0.05$), further suggesting that overall effects of radiation on FA in metrical traits of *A. aquaticus* were limited. However, for the meristic segment asymmetry trait both significant differences between sites of varying contamination and a positive relationship between FA and radiation was recorded (see Figure 3.13E), suggesting that this trait may have been affected by chronic radiation exposure.

Regression analysis found no significant relationship between any of the environmental variables and FA (Regression analysis, df = 1,110, $p > 0.05$). This suggests that the measured confounding factors did not influence observed FA differences in *A. aquaticus* in 2004.

ii) *Eriocheir japonica*

In *E. japonica*, no significant differences in levels of FA between sampling sites were recorded, suggesting that radiation did not impact developmental stability in this organism. This was further emphasised by the lack of a relationship between individual traits and total dose rate (see Figure 3.14). No significant relationships were recorded between dose rate and FA1 in any of the five measured morphometric traits (see Figure 3.14, Spearmans rho, $p > 0.05$). Furthermore, analysis of the relationship of composite FA values with dose rates recorded no significant relationship (Spearmans rho = 0.05, $p > 0.05$). No significant effect of any of the measured environmental characters on FA was recorded (Regression analysis, df = 1, 90 $p > 0.05$).

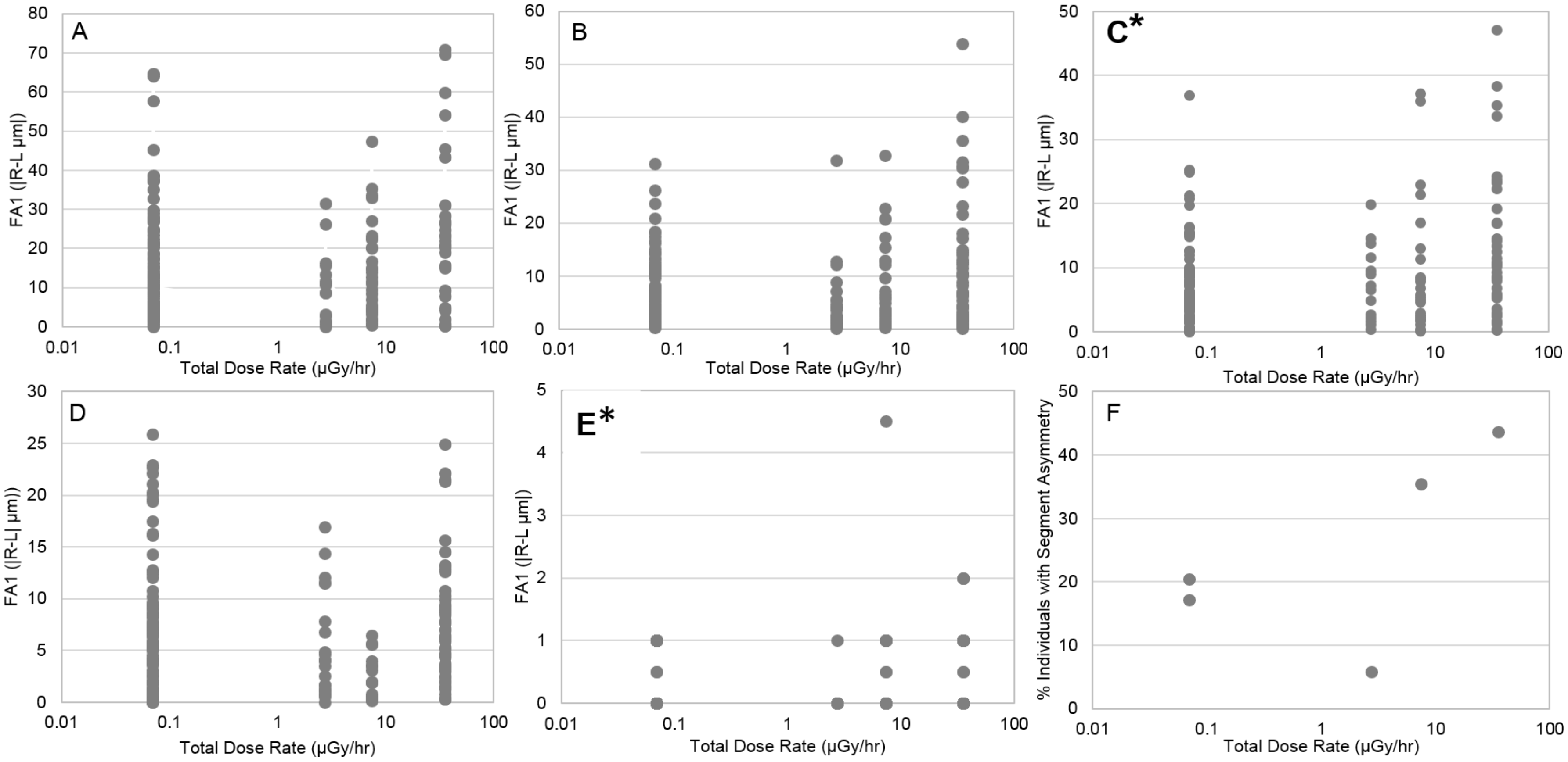


Figure 3.13 – Relationship between fluctuating asymmetry and radiation dose rate in five morphometric traits in *A. aquaticus* collected in 2004/2005. A) First Antennae Length. B) Propodos Width C) Merus Width D) Carpus Width E) Number of Segments F) % Individuals exhibiting segment asymmetry. * Indicates a significant relationship (Spearmans rho, $p < 0.05$).

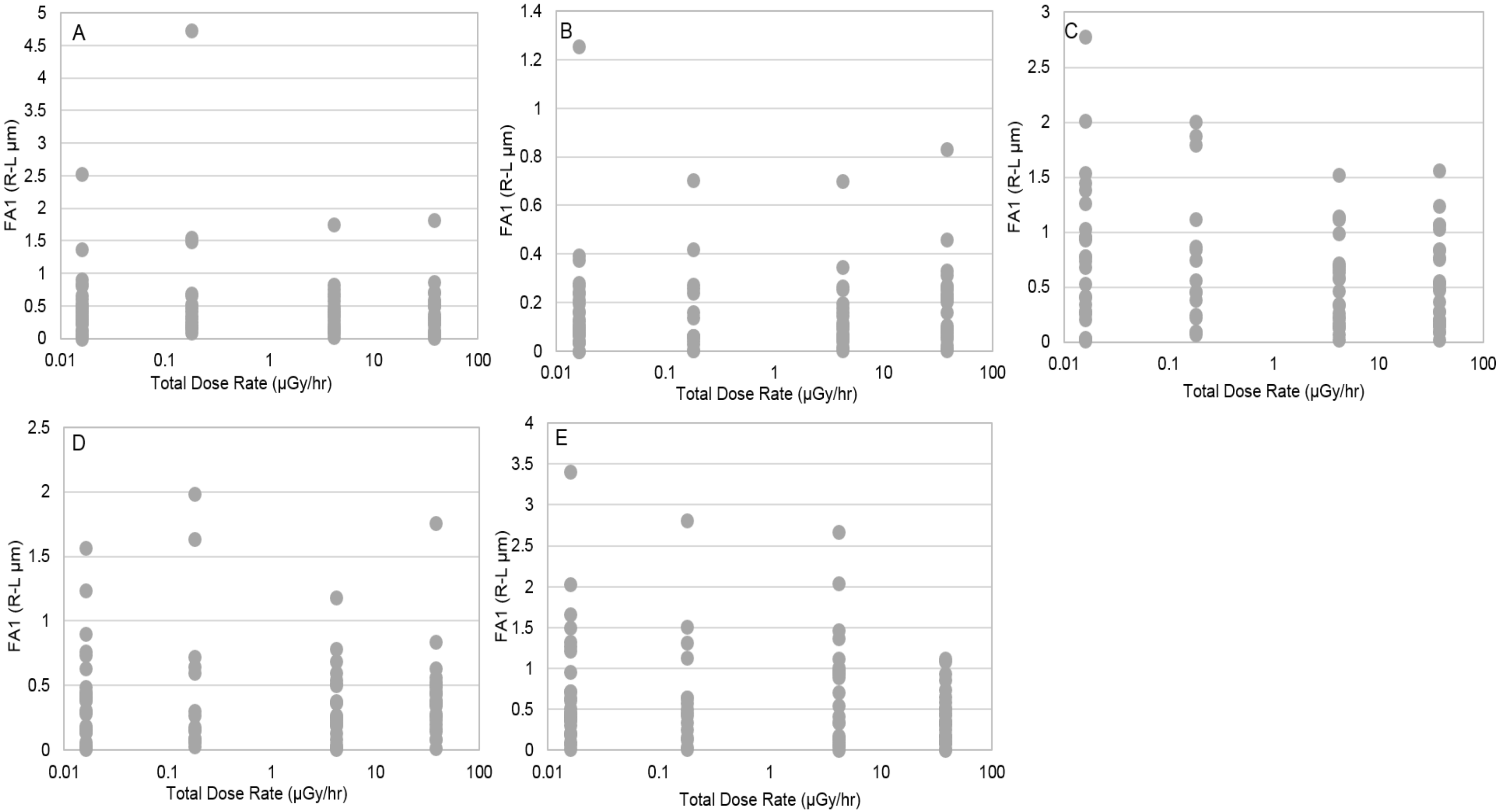


Figure 3.14 – Relationship between fluctuating asymmetry in five morphometric traits in *Eriocheir japonica* and total dose rates at five sites of varying contamination at Fukushima. A) Merus Length B) Merus Width C) Carpus Length D) Carpus Width and E) Propodos Length.

3.4 Discussion

The present study hypothesised that chronic radiation exposure would lead to elevated fluctuating asymmetry in populations of *Asellus aquaticus* and *Eriocheir japonica* at Chernobyl and Fukushima respectively. No significant relationship between radiation dose rate and FA was found in the 2015 *A. aquaticus* samples or *E. japonica*, leading to rejection of this hypothesis. It was further hypothesised that a greater effect of radiation on FA would be observed in samples collected closer to the time of the accident (2004/2005). Samples of *A. aquaticus* collected in 2004/2005 showed significant relationships between radiation dose rate and FA for two of the five measured traits, suggesting a slight effect on FA. However, the lack of a relationship between two other measures of FA (FA2 and CFA2, See Sect 3.3) and radiation dose rates within these samples suggests only limited effects of chronic radiation exposure on FA in *A. aquaticus*. Therefore, only limited support for this hypothesis was found within the data.

These findings are different to those in the available literature which suggest an unequivocal increase in FA in response to radiation exposure. This has been documented for a range of biota including fish (Lajus et al., 2014; Zakharov et al.,1996), small mammals (Gileva & Nokhrin, 2001; Oleksyk et al., 2004), birds (Møller, 1993), insects (Møller, 2002) and plants (Møller, 1998). Studies of aquatic invertebrates from the Chernobyl region further support an increase in FA as a result of radionuclide exposure. Yavnyuk et al., (2009) measured FA in two species, the zebra mussel *Dreisenna polymorpha* and floating pondweed, *Potamogeton natans*. The authors recorded a 25-fold increase in asymmetry of *D. polymorpha* collected from the Chernobyl cooling pond compared to reference areas within the Dnieper River. A smaller increase in *P. natans* FA of 1.4-fold compared to samples collected from the Kiev reservoir was documented.

Molluscs have been demonstrated to accumulate and retain radionuclides at concentrations orders of magnitude higher than other aquatic biota (Frantsevich et al., 1996; Jaeschke et al., 2015) and are susceptible to elevated dose rates. For example, Gudkov et al., (2016) recorded dose rates of 350 - 420 µGy/hr in the freshwater snail, *Lymnaea stagnalis* collected from Glubokoye lake over a study period from 1998 – 2014. Dose rate estimates to *Asellus aquaticus* and *Eriocheir japonica* were an order of magnitude lower in the present study. The lower dose rates may not have been to sufficient to cause a discernible increase in developmental instability as indicated by FA. Furthermore, all of the previously mentioned studies were conducted closer to the time of the accident (mid to late 1990's) where dose rates would have been higher as compared to the present study. Similarly, over longer time scales organisms may have adapted to chronic radiation exposure. This may explain the lack of effect in the present study as compared to the existing literature.

i) Asellus aquaticus

Amongst the *A. aquaticus* samples collected in 2004/2005, a significant relationship between segment asymmetry and radiation dose rate was recorded. Similarly amongst 2015 samples, a positive relationship between this trait and dose rate was recorded, though this was non-significant. This suggests that this trait may be a sensitive indicator of radiation-induced developmental stress in *A. aquaticus* as has been previously demonstrated following temperature stress by Savage & Hogarth (1999). The change in relationship between segment asymmetry and dose rate between 2015 and 2004/2005 samples may indicate adaptation of *A. aquaticus* to chronic radiation stress. Assuming *A. aquaticus* to be univoltine (Brattey, 1986), populations would have undergone approximately eleven generations between the two sampling periods and thirty generations following the Chernobyl accident in 2015. Studies have demonstrated adaptation of aquatic invertebrate populations exposed to a range of pollutants over relatively short timeframes (Goussen et al., 2015; Hochmuth et al., 2015; Sun et al., 2014). For example, Sun et al., (2014) found evidence for adaptation to tributyltin oxide (TBTO) in the sexually reproducing marine copepod, *Tigriopus californicus* in seven generations. Elevated mutation frequencies have been reported in a range of organisms from Chernobyl (See Møller & Mousseau, 2015 for meta-analysis). However, studies of potential adaption to chronic radiation stress are limited and at present inconclusive (e.g. Geras'kin et al., 2011; Klubicová et al., 2010).

Another possibility for the reduced FA over time in the segment asymmetry trait between 2015 and 2004 may be change in dose rates over this period owing to radioactive decay. Dose rates in 2004 were 35.1 µGy/hr at the most contaminated site, as compared to 27.1 µGy/hr in 2015. A threshold for induction of FA may exist between these two dose rates, thus explaining the lack of a significant relationship between dose rate and segment FA in 2015.

Significantly greater mean asymmetry was observed at Svyatoye-3 in 2015 relative to all sites excluding Dvorische and Glubokoye lakes. Differences in asymmetry were not found to be related to any of the measured environmental parameters using regression analysis. Elevated developmental instability at this site may be due to a biotic stressor, for example an increased parasitic burden. A number of studies have demonstrated positive correlations between parasite burden and FA (e.g. Alibert et al., 2002; Bonn et al., 1996; Cuevas-Reyes et al., 2011). *Asellus aquaticus* is an intermediate host to acanthocephalan parasites (Brattey, 1986) and has also been demonstrated to harbour *Wolbachia*, a parasitic intracellular bacteria (Bouchon et al., 1998). Acanthocephalan infection has been linked to an increase in FA in another freshwater aquatic invertebrate species, *Gammarus pulex,* which occupies an overlapping ecological niche (Alibert et al., 2002; Graça et al., 1994). Furthermore, sex ratios of *A. aquaticus* at Svyatoye-3 were skewed in the favour of females relative to other sites (0.31:0.69 males to females, data not shown), which could be indicative of *Wolbachia* infection, since this parasite typically causes feminization of host organisms (Rigaud et al., 1999). Future research should adopt a PCR-based approach to screening populations of *A. aquaticus* for *Wolbachia* infection.

ii) *E. japonica*

This study represents the first assessment of radiation effects on crustaceans at Fukushima. The majority of studies at Fukushima to date have focused solely on the accumulation of radionuclides by aquatic organisms (e.g. Wada et al., 2016a,b; Yoshimura & Akama, 2014) and no studies to date have documented effects on aquatic invertebrates. A number of studies have documented morphological effects of radiation exposure on terrestrial invertebrates at Fukushima at lower doses than in the present study, though FA was not directly used. Hiyama et al., (2012) reported morphological abnormalities in the pale grass blue butterfly, *Zizeeria maha* at low dose rates of 1.6 µSv/hr. Similarly, increased morphological abnormalities in the gall-forming aphid *Tetraneura sorini* were recorded by Akimoto (2014) at ground dose rates of 6.0 µSv/hr. Though differences in the species biology and endpoint used preclude an accurate comparison, terrestrial invertebrates are often considered to have similar radiosensitivity as compared to aquatic invertebrates. For example, DCRLs for the ICRPs reference crab are the same as those for both the reference earthworm and bee (ICRP, 2012). Therefore, when considering environmental risk assessments these organisms would be anticipated to display negative effects at the same dose rates. Differences in the response to radiation between the present and aforementioned studies suggest that the development of more species-specific dose benchmarks may be necessary.

Though no significant differences in overall levels of fluctuating asymmetry were recorded in *E. japonica,* highly significant differences between traits were observed (see Section 3.3.4ii). This was due to significantly lower FA in the merus length and width of the fourth paraeopod (final pair of walking legs) as compared to all other traits. In decapod crustaceans, the back legs play important roles in postural adaptation (Clarac, 1981). Furthermore, the rear walking legs are often used for mating or aggression behaviours in crabs (Cameron, 1966; Degoursey & Auster, 1992). Given the important role of the rear walking legs in crabs, stronger pressure may be exerted on this trait to remain symmetrical thus explaining the lower FA as compared to other traits.

In the present study, calculated dose rates from ERICA to *E. japonica* ranged from 0.016 to 38.1 µGy/hr. This represents the first dose assessment to a commercially and ecologically important freshwater crab species at Fukushima. A number of studies have assessed doses to marine biota following the accident (e.g. Johansen et al., 2015; Kryshev et al., 2012; Vives i Batlle et al., 2014), generally recording doses below levels of expected harm and a significant decline in radioactivity levels over time. However, despite a number of studies documenting persistent high levels of radioactivity in freshwater biota at Fukushima (e.g. Mizuno & Kubo, 2013; Wada et al., 2016a), dose rates have not been calculated. Furthermore, though a number of freshwater aquatic invertebrates have been monitored for radiocaesium contamination by the Japanese Ministry of Labour, Health & Welfare (MLHW, See Okamura et al., 2016), data is generally lacking for the most contaminated areas in the immediate vicinity of the FDNPP. For example, for the model organism used in the present study, radionuclide contamination data for only a single *E. japonica* individual collected in the Fukushima region is provided in the MLHW dataset. Though below the level where significant harm is expected (Larsson, 2012), the finding that dose rates remain high six years post-accident has important consequences for the long-term management of freshwater systems at Fukushima.

There are a number of limitations associated with the methods used to calculate dose rates to crabs in the present study. For example, dose calculations were based solely on measurements of radiocaesium in sediment and water. Recent studies have demonstrated the importance of dietary accumulation of radiocaesium in crabs ingesting contaminated benthic fauna such as polychaete worms (Wang et al., 2016). Assimilation of Cs by polychaete worms and subsequent consumption by organisms was found to account for > 90% of the Cs body burden in the Asian shore crab, *Hemigrapsus sanguineus* (Wang et al., 2016). *E. japonica* is known to be omnivorous, feeding both on vascular plant detritus and macroinvertebrates (Kobayashi, 2009). Future research should therefore focus on assessing radiocaesium contamination in a range of macroinvertebrate prey organisms coupled with stable isotope analysis to infer dietary preferences of *E. japonica* at these sites. This would enable a robust understanding of radiocaesium dynamics and doses to crabs at these localities.

3.5 Summary & Limitations

The present study comprehensively analysed the effects of chronic radiation exposure on FA in two species utilising the full gradient of contamination at Chernobyl and Fukushima. Owing to numerous studies detailing positive relationships between radiation dose rates and FA, we hypothesised that levels of FA would be increased in populations of *A. aquaticus* and *E. japonica* at higher dose rates. Overall, no significant relationship between FA and radiation dose rate was found leading to rejection of this hypothesis, though evidence for slight effects in samples of *A. aquaticus* collected in 2004 was presented. This suggests that at present, either chronic radiation exposure at Chernobyl and Fukushima is not causing effects on the development of *A. aquaticus* and *E. japonica* or effects are so subtle as to be undetectable using the FA method. A number of studies have found no increase in FA in response to a range of stressors, highlighting the limitations of FA as an indicator (Bjorksten et al., 2000; Floate & Fox, 2000; Vangestel & Lens, 2011; Zverev et al., 2018).

FA – stress relationships are known to be confounded by a number of factors including trait choice, degree of measurement error and specific stressor studied. Every study of the effects of radiation on FA to date have demonstrated a positive stress-FA relationship, therefore FA is an appropriate indicator for this particular stressor. Similarly, the contribution of measurement error was assessed in the present study and was not found to significantly impact observed FA. Though it is possible that the traits selected in *A. aquaticus* and *E. japonica* may have been inadequate to detect stress-FA relationships, traits were preferentially selected on the basis of previous studies with confirmed efficacy as stress indicators.

In order to conclusively assess the impact of the Chernobyl and Fukushima accidents on the developmental stability of *A. aquaticus* and *E. japonica*, an assessment of levels of FA prior to the nuclear accidents would be necessary. The lack of pre-accident samples is therefore one of the key limitations of the present study, however such samples were not available. Further, the use of 'museum' specimens in FA studies has been criticised (Swaddle et al., 1994) owing to the selection of developmentally normal specimens and the deterioration of trait quality over time. To try and address this limitation, samples collected slightly closer to the time of the Chernobyl accident were analysed, within which only slight effects on FA were recorded.

Many of the available studies of the effects of radiation on FA in non-mammalian organisms utilised only a single contaminated and reference site (e.g. Lajus et al., 2014; Møller, 2002). By comparison, this study utilised the full range of contamination in aquatic systems in the Chernobyl and Fukushima regions providing a comprehensive analysis of the effects of radiation on FA. The greater range of dose rates in the present study strengthens any observed associations between radionuclide contamination and FA and may account for the differences between the present and previous studies of developmental instability in radiation exposed organisms.

Chapter 4

Effects of Chronic Radiation Exposure on the Reproduction of *Asellus aquatics* at Chernobyl

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4.1 Introduction

4.1.1 Radiation Effects on Reproduction in Natural Populations

Many studies have focused on the effects of radiation on the reproduction of natural populations, due to the clear potential for higher-level impacts. In birds, many studies have reported deleterious effects of radiation exposure at Chernobyl and more recently Fukushima. Møller et al., (2005) reported reduced clutch sizes, lower hatching success and a smaller brood size of hatchlings in populations of barn swallows, *Hirunda rustica*, at at Chernobyl as compared to control areas. Similarly in the great tit, *Parus major*, reduced antioxidants in eggs in populations inhabiting contaminated areas at Chernobyl was associated with a reduction in hatching success (Møller et al., 2008).

Recent work has demonstrated negative effects of Fukushima-derived radiation on the reproductive success of the goshawk, *Accipiter gentilis* (Murase et al., 2015). Field observations were performed over a period of 22 years and reproductive performance was found to decline in the three years following the nuclear accident as compared to pre-accident samples. Bonisoli-Alquati et al., (2015) recorded a reduction in the number of juvenile barn swallows at highly contaminated areas in Fukushima, which was suggested to be related to suppressed reproduction in these populations. In murine rodents, a number of studies reported perturbations to reproduction and elevated embryonic mortality following the Chernobyl accident (Krylova et al., 1992; Testov & Taskaev, 1990), though effects appeared to be limited to the initial phase after the accident. A number of other studies have studied effects of radiation at Fukushima on male fertility and have been summarised in Section 2.1.

In aquatic systems, studies of the effects of chronic radiation exposure on reproduction are more limited. In a range of fish species including roach, *Rutilus rutilus*, perch, *Perca fluviatilis* and the goldfish *Carassius auratus*, gonadal abnormalities including asymmetry and oocyte resorption were positively correlated with contamination levels over a period from 1992 – 2005 (Belova et al., 2007). Subsequent studies of five species of fish collected in 2007 and 2008 demonstrated the persistence of deleterious effects greater than 20 years post-accident (Belova & Emel'yanova, 2009). Similarly, a range of morphological abnormalities in the reproductive system of the common reed, *Phragmites australis*, inhabiting highly contaminated lakes was recorded by Nurgudin et al., (2009).

For aquatic invertebrates, only a single study exists to the author's knowledge which does not directly quantify reproduction. Tsytsugina & Polikarpov (2003) studied populations of three species of oligochaete worm *Deis obtusa, Nais pseudobtusa* and *Nais pardalis* at a Chernobyl contaminated lake and a reference lake. An increase in the percentage of individuals with developing germ cells at the contaminated site in two of the three analysed species was recorded, which was associated with an increase in chromosomal aberrations. Asexual reproduction is the predominant mode of reproduction in these oligochaete species, therefore sexual reproduction is considered to be triggered in unfavourable environmental conditions, enhancing the adaptive potential of the population. The authors therefore attributed the increase in sexual reproduction to elevated radionuclide contamination, though the effect was not consistent across all three species. This study only assessed a single contaminated and reference site, thereby the observed effect on mode of reproduction may have been due to another factor than radiation. Furthermore, though ^{90}Sr contamination in sediment was assessed, no dose rates were calculated and reproductive output was not quantified. The present study aimed to address the limitations of the previous by utilising a gradient of radionuclide contamination to gain a robust understanding of the effects of radiation on reproduction in *Asellus aquaticus*.

4.1.2 Reproduction in Asellus aquaticus

Similar to the amphipods *E. marinus* and *G. pulex* (see Chapter 2), *A. aquaticus* displays a precopulatory mating phase wherein the male guards the female for a period before mating. The typical duration of this phase for *A. aquaticus* is between six to eight days (Manning, 1975). Following moulting, the female becomes ovigerous and copulation occurs. The eggs are carried by the female within a marsupium (brood pouch) for an approximate period of 15 days (Williams, 1960). During this period eggs undergo five stages of development according to the classification of Holdich (1968) on the marine isopod, *Dyamane bidentata*. Following this brooding period, live offspring are released. The life history of *A. aquaticus* varies geographically, with three general patterns described. Populations inhabiting warmer Mediterranean regions reproduce continuously throughout the year (Tadini & Valentino, 1969), whilst *A. aquaticus* inhabiting colder regions reproduce in summer only. Popluations breeding in winter only have also been described (e.g. Southern France, Vandel 1926). No data exists regarding the life history of *A. aquaticus* in the Ukraine or Eastern Europe. In the majority of recorded cases, *A. aquaticus* is semelparous, reproducing once during its lifetime (Chambers, 1977; Gruner, 1965; Murphy & Learner, 1982; Steel, 1961).

A number of studies have considered the effects of pollutants on the reproduction of *A. aquaticus* in contaminated environments. Maltby (1991) studied populations above and below a coal mine effluent, and found that *A. aquaticus* downstream of the effluent had lower reproductive effort (defined as mg offspring/mg female) as compared to upstream. Significantly fewer but larger offspring were produced downstream, which the author attributed to life history modification due to chronic pollutant exposure. Populations exposed to mine effluents were cultured in the laboratory for two years and were found to retain life history modification, suggesting a genetic basis. Similarly, Tolba & Holdich (1981) recorded a lower fecundity in *A. aquaticus* individuals collected from sites with high degrees of pollution. In laboratory studies of the effects of radiation on crustacean reproduction (see Sect 1.3.4 for in-depth review), perturbations to the timing of reproduction have been recorded (Alonzo et al., 2008a; Parisot et al., 2015; Won & Lee, 2014). Similarly, in terrestrial isopods inhabiting metal contaminated environments early reproduction was proposed to be the mechanism for observed reduced fecundity (Donker, 1992; Donker et al., 1993).

4.1.3 Aims & Objectives

To date, no study has assessed the effects of chronic low-dose radiation exposure on the reproduction of natural crustacean populations. Based on the studies described above and the known deleterious effect of radiation on crustacean reproduction in the laboratory (see Section 1.3.4), it was hypothesised that:

- *Asellus aquaticus* individuals inhabiting contaminated areas at Chernobyl would display a reduced reproductive output and altered timing of reproduction.

In order to test this hypothesis, *A. aquaticus* individuals were collected from six lakes along a gradient of radionuclide contamination in Belarus and the Ukraine and fecundity, brood mass and maternal weight were assessed. Maternal weight was used as a proxy of reproductive timing (Donker et al., 1993).

4.2 Materials & Methods

4.2.1 Field Sampling & Collection of Asellus aquaticus

Samples of *A. aquaticus* were collected in May – June of 2015 and 2016 according to the methods described in 3.2.1. For the Belarusian lakes in 2016 (Svatoye #3, Stoyecheye and Dvorische), samples were collected by colleagues at the Belarusian National Academy of Sciences according to the same protocol. Table 4.1 shows the measured environmental parameters and sampling date for both years. Dose rates for 2016 were decay corrected from the calculated values described in Section 3.2.2.

Table 4.1 – Sampling date and measured environmental characters at six lakes along a gradient of radionuclide contamination in Belarus and the Ukraine. Values for environmental parameters are presented as mean ± SD for values taken at three sub-sites of each lake.

Site	Sampling Date	Dose Rate (µGy/hr)	Temperature (°C)	Oxygen Saturation (%)	pH	Conductivity (µS/cm)
Gorova	11/6/2016	0.064	22.4 ± 0.05	113 ± 16.2	8.60 ± 0.02	256 ± 0.41
	23/6/2015	0.064	22.2 ± 0.05	185 ± 26.5	9.69 ± 0.02	179 ± 0.30
Dvorische	29/05/2016	0.691	23.2 ± 0.06	80 ± 1.17	7.60 ± 0.17	197 ± 0.15
	11/06/2015	0.786	23.7± 0.06	68.9 ± 1.00	7.82 ± 0.17	200 ± 0.00
Stoyecheye	27/05/2016	0.774	22 ± 0.05	102 ± 2.00	8.30 ± 0.02	241 ± 1.48
	08/06/2015	0.872	24.1 ± 0.06	89.4 ± 1.76	8.70 ± 0.02	230 ± 1.41
Svatoye 3	24/05/2016	2.03	20.1 ± 0.23	92 ± 1.80	8.00 ± 0.15	114 ± 0.70
	05/06/2015	2.2	23 ± 0.70	81.6 ± 1.60	7.8 ± 0.14	122 ± 0.75
Yanovsky Crawl	05/06/2016	20.42	20.2 ± 0.11	140 ± 2.90	9.00 ± 0.04	265 ± 0.97
	19/06/2015	20.6	23.3 ± 0.11	109 ± 2.25	9.40 ± 0.04	275 ± 1.00
Glubokoye	03/06/2016	26.4	23.6 ± 0.06	112 ± 14.10	7.60 ± 0.18	199 ± 1.22
	16/06/2015	27.1	24.9 ± 0.06	66.3 ± 8.32	7.92 ± 0.19	185 ± 1.14

4.2.2. Reproductive Output in A. aquaticus

Adult (> 3mm in length, Hasu et al., 2007) *A. aquaticus* individuals were first sexed following Bertin et al., (2002) by analysis of the pleopods. Individuals were measured and weighed using the Leica Application Suite (v 4.5) and a Kern ABT 120-5DM (DE) analytical fine balance with a precision of ± 0.02 mg respectively. Embryos were removed from the marsupium of gravid females (individuals bearing eggs) using a glass Pasteur pipette and photographed individually using a Leica DFC310 camera. Individuals were then reweighed to provide an estimate of the total weight of the brood. Embryos were staged following the method of Holdich (1968). Table 4.2 displays the number of gravid individuals that were analysed over the two years of sampling.

Table 4.2 – Numbers of gravid and non-gravid *A. aquaticus* individuals collected over two sampling years at six lakes along a gradient of radionuclide contamination

Sample Site	Sampling Year	Gravid	Non-Gravid	Proportion Gravid (%)
Gorova	2016	60	81	42.56
	2015	19	33	36.54
Dvorische	2016	0	55	0
	2015	14	31	31.11
Stoyecheye	2016	9	34	20.93
	2015	3	44	6.38
Svatoye 3	2016	7	6	53.85
	2015	20	25	44.44
Yanovsky Crawl	2016	32	53	37.65
	2015	16	28	36.36
Glubokoye	2016	83	35	70.34
	2015	31	42	42.47
Total		**294**	**467**	**38.63**

4.2.3 Statistical Analyses

Differences in brood sizes and weights between sites were tested using linear mixed effects models using the nlme package (Pinheiro et al., 2016) in R Studio Version 1.01 (R Studio Team, 2016). Sampling site, year and developmental stage of the brood were used as fixed effects and maternal weight as a random effect. In crustaceans, larger females typically produce greater numbers of eggs (e.g. Oh & Hartnoll, 2002). Maternal weight is therefore typically used as a covariate in analyses of crustacean brood sizes (e.g. Ford et al., 2003). Owing to heterogeneity in the relationship between maternal weight and egg numbers between lakes in the present study (see Figure 4.1), a random slopes and intercepts model was used. Models were fit using maximum likelihood methods and validated by analysis of residuals at each level of the random effect. Residuals were visually inspected for homoscedasticity and tested for normality using the shapiro.test function. Post-hoc multiple comparisons were used with Tukey contrasts via the multcomp package (Hothorn et al., 2008).

Differences in the percentage of adult females between sites of varying contamination and sampling years was tested using binary logistic regression with the glm function. Female reproductive status (gravid = 1, non- gravid = 0) was used as the binary dependent variable where site and sampling year were predictors. Owing to a significantly non-normal dataset, differences in the body mass of gravid *A. aquaticus* between sample sites were tested using a Kruskal-Wallis and post-hoc Dunn's test with Benjamini-Hochberg correction via the dunn.test package (Dinno, 2015). Relationships between measured environmental characters and reproductive parameters were tested using linear regression. Where assumptions of regression were violated, Spearman's rho was used. All statistics were conducted in R Studio (R Core Development Team, 2016).

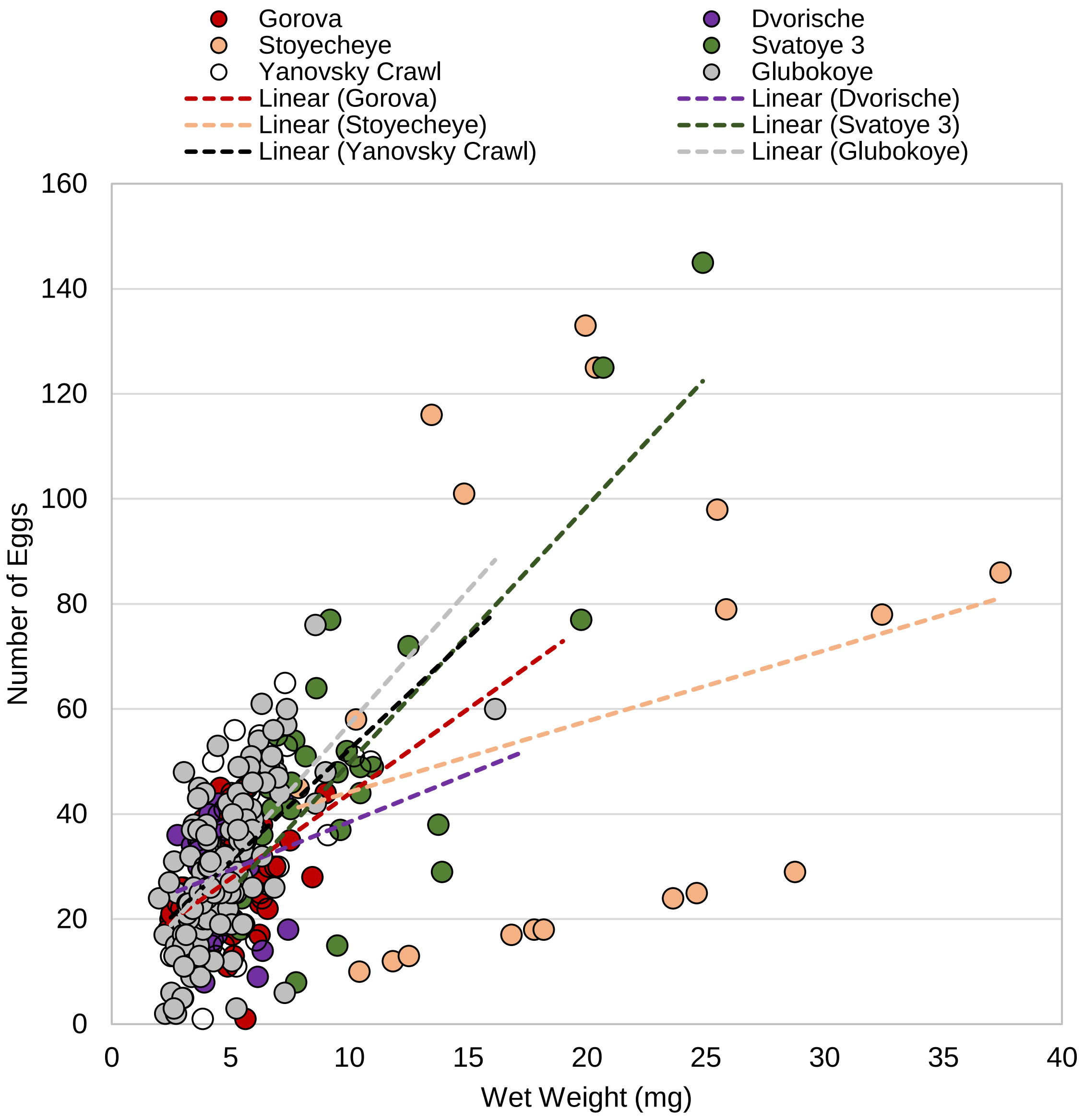

Figure 4.1 – Heterogeneity in the relationship between wet weight and number of eggs in *Asellus aquaticus* at six lakes along a gradient of radionuclide contamination at Chernobyl. Significant positive relationships (Regression analysis, $p < 0.05$) were recorded for each weight x eggs combination excluding Stoyecheye ($p > 0.05$).

4.3 Results

4.3.1 Proportion of Breeding Females

The proportion of gravid females was not related to radiation dose rate (Figure 4.2, linear regression, $F_{1, 10} = 3.262$, $r^2 = .246$, $p = .101$). The number of adult *A. aquaticus* with broods was significantly different between sites however (logistic regression, $\chi = 8.65$, df = 5, $p < .001$), but not between sampling years ($\chi = 1.85$, df = 1, $p = .065$). The most contaminated site, Glubokoye had the greatest proportion of females with broods over two years of sampling (Mean ± SE, 56.4 ± 13.9%), significantly greater than all sites excluding Svatoye 3 (49.1 ± 4.7%, Post-hoc Tukey's contrast, $p > 0.05$). The lowest proportion of females with broods was recorded at Stoyecheye (10.3 ± 7.3 %). The raw data for the number of gravid females is shown in Table 4.2. The proportion of breeding females was not correlated with any of the measured confounding factors; dissolved oxygen ($F_{1, 10} = 0.3447$, $r^2 = .033$, $p = .570$), water temperature ($F_{1, 10} = .357$, $r^2 = .034$, $p = .564$), conductivity ($F_{1, 10} = .925$, $r^2 = .085$, $p = .359$) and pH ($F_{1, 10} = .107$, $r^2 = .011$, $p = .750$).

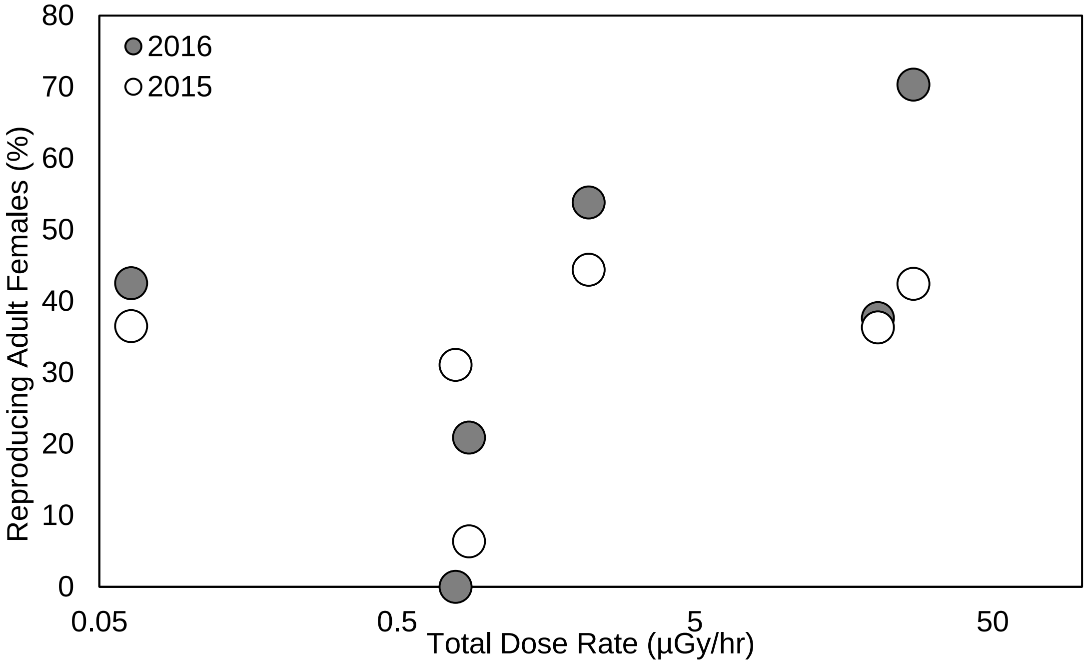

Figure 4.2. – Scatterplot of the relationship between the proportion of reproducing adult (>3 mm) female *A. aquaticus* in summer 2015/2016 and total dose rate at six lakes in Belarus and the Ukraine.

4.3.2 Fecundity

A strong positive relationship between number of eggs and female wet weight was recorded (see Figure 4.1), therefore weight-normalised values were used for comparisons across sampling sites. The results of the linear mixed model for both fecundity and brood mass are shown in Table 4.3. The number of eggs produced by female *Asellus aquaticus* did not vary between sampling year ($F_{1,80}$ = 7.5, p = .740) or with the developmental stage of the brood ($F_{1, 80}$ = .602, p = .502). The greatest number of eggs was produced at Glubokoye lake over the two sampling years (Mean ± SD, 6.477 ± 2.259 eggs per mg maternal weight, See Figure 4.3) with the fewest eggs being produced at Stoyecheye lake (4.811 ± 1.824 eggs per mg maternal weight), however no significant effect of sampling site on brood sizes was recorded ($F_{1, 80}$ = 2.402, p = .494). This was further emphasised by the lack of relationship between total dose rate and weight-normalised brood sizes (see Figure 4.4, Spearman's rank-order correlation, rho = .008, p = .877).

Table 4.3 Results of the linear mixed model for number and mass of broods produced by *Asellus aquaticus* along a gradient of radionuclide contamination at Chernobyl.

Model	Log Likelihood	Effect	t value	SE	p value
Egg Number	-481.2	Site	0.688	0.081	0.494
		Development Stage	0.674	0.070	0.502
		Sampling Year	-0.333	0.190	0.740
		Site* Developmental Stage	-1.162	0.016	0.249
		Site*Sampling Year	1.769	0.044	0.081
Egg Mass	-562.2	Site	0.902	0.093	0.370
		Sampling Year	0.775	0.234	0.441
		Developmental Stage	3.246	0.087	0.002
		Site*Sampling Year	-0.184	0.052	0.855
		Site*Developmental Stage	-1.051	0.019	0.297

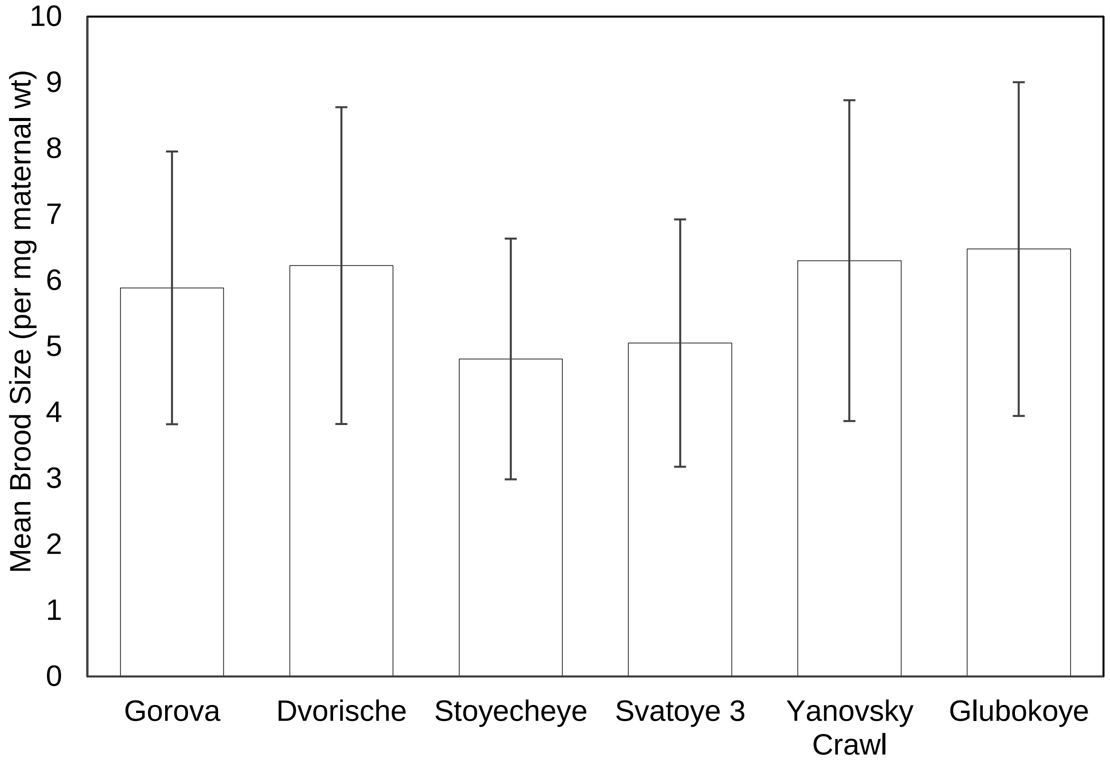

Figure 4.3 - Mean brood sizes normalised to maternal weight in *Asellus aquaticus* from six sites along a gradient of radionuclide contamination in Belarus and the Ukraine. Sites are plotted in order of increasing contamination from left to right. Error bars represent standard deviation.

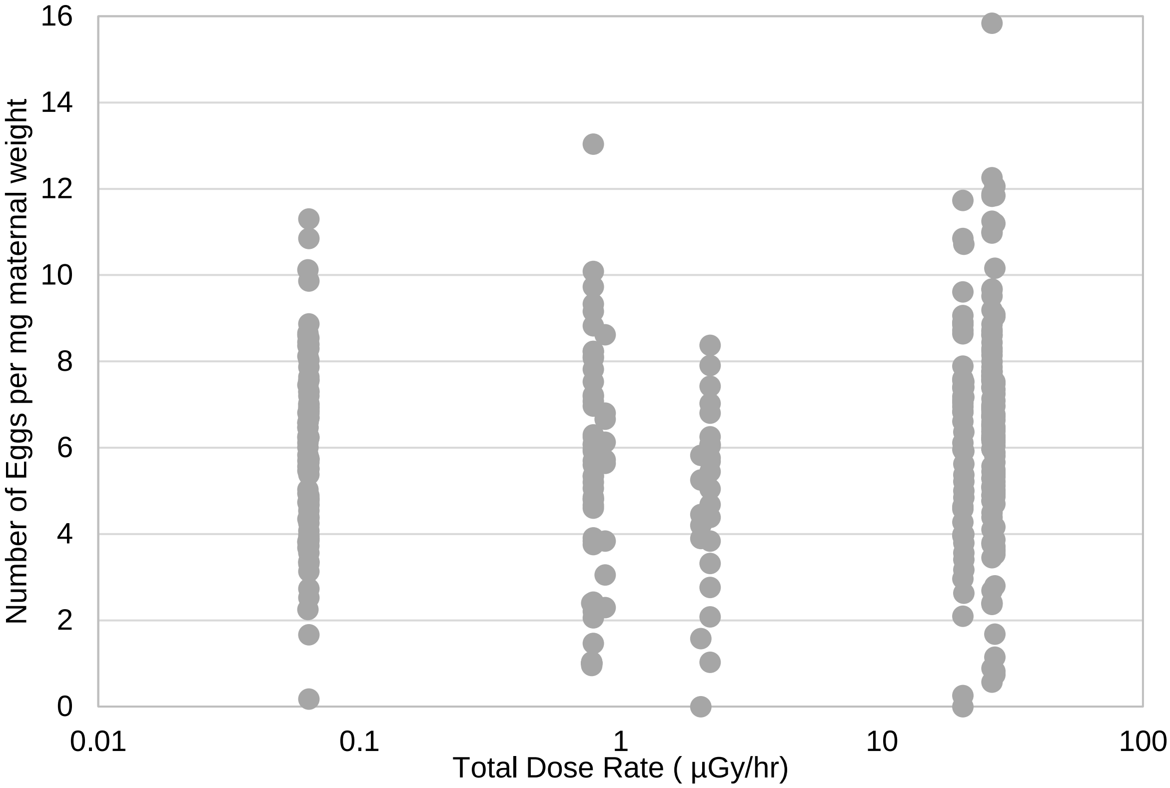

Figure 4.4 – Relationship between weight-normalised brood sizes and total dose rate in *Asellus aquaticus* along a gradient of radionuclide contamination.

4.3.3 Brood Mass

Brood mass did not vary significantly between sampling years ($F_{1, 70}$ = 3.653, p = .441) or between sampling sites of varying radionuclide contamination ($F_{1, 70}$ = .562, p = .456, see Figure 4.5). Developmental stage was found to have a significant effect on the mass of the brood however (see Table 4.3, $F_{1, 70}$ = 25.060, p = .0018). This was owing to a significantly (Post-hoc Tukey contrasts, p = .0018) greater mass of broods in the final stage of development (Mean ± SE, 2.847 mg ± .590) compared to the first stage of development (1.045 mg ± 0.073). This effect was independent of sampling site as indicated by the non-significant interaction between sampling site and developmental stage (see Table 4.3, $F_{1, 70}$ = .642, p = .426).

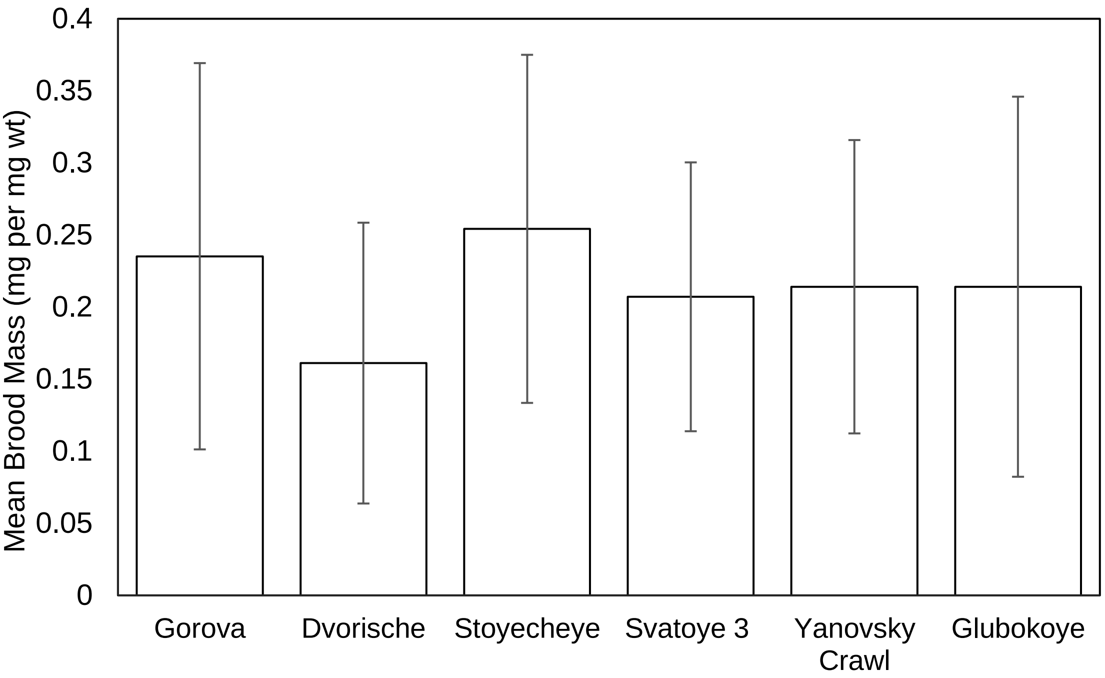

Figure 4.5 - Mean brood mass normalised to maternal weight of *A. aquaticus* collected from six sites along a gradient of radionuclide contamination. Sites are plotted in order of increasing contamination from left to right. Error bars represent standard deviations.

4.3.4 Maternal Body Mass

No significant differences in the body mass of gravid *Asellus aquaticus* were recorded between sampling years (Kruskal-Wallis test, χ^2 = .347, df = 1, p = .558). Significant differences in maternal body mass were recorded between sampling sites (Kruskal-Wallis test, χ^2 = 109.4, df = 5, p = .000), owing to a significantly greater mass of *A. aquaticus* at Stoyecheye (18.938 ± 6.904 mg) and Svatoye 3 (11.730 ± 4.735 mg) compared to all other sites (see Figure 4.6, Dunn's test, p = .000). Differences in body mass were not related to total radiation dose rate (Spearman's rank-order correlation, rho = -.081, p = .129) or any of the other measured environmental variables; conductivity (rho = -.062, p = .245), temperature (rho = -.077, p = .149), pH (rho = .108, p = .449) and dissolved oxygen (rho = .878, p = .878).

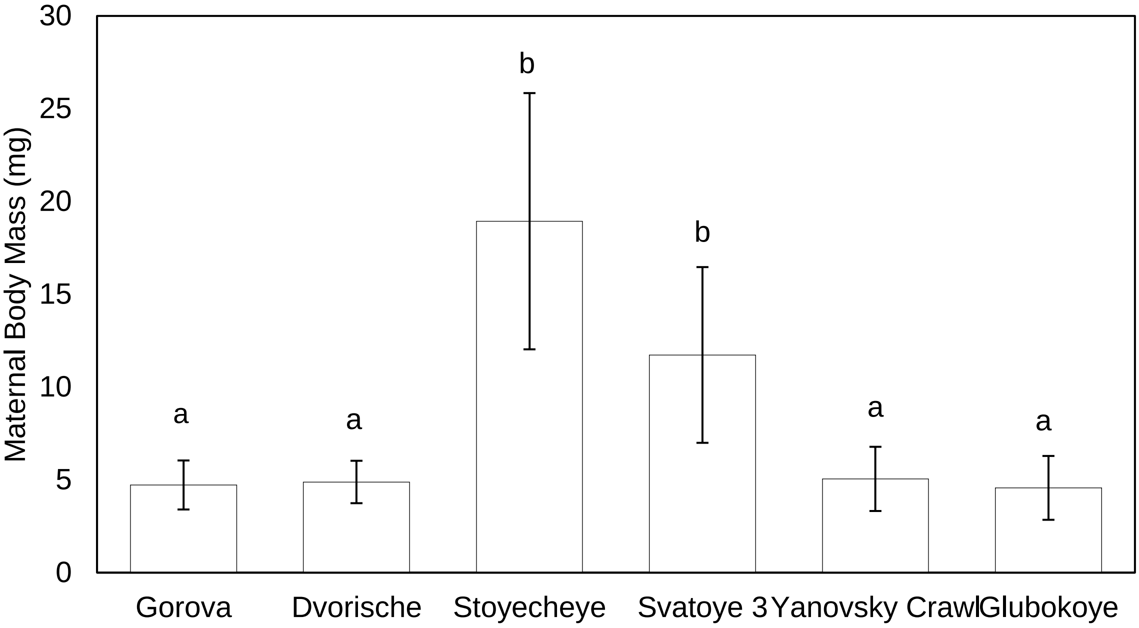

Figure 4.6. – Body mass of gravid *A. aquaticus* from six sites along a gradient of radionuclide contamination in Belarus and the Ukraine. Matching letters represent no significant difference, difference letters represent significant differences (Dunn's test, $p < 0.05$). Error bars are ± SD.

4.4 Discussion

The present study hypothesised that populations of the isopod crustacean *Asellus aquaticus* chronically exposed to ionising radiation at Chernobyl would display reduced reproductive output and altered timing of reproduction. In order to test this hypothesis, gravid females were collected from six sites along a gradient of radionuclide contamination in areas impacted by Chernobyl in 2015 and 2016. Reproductive output was assessed and weight of gravid females was used as a proxy of timing of reproduction. No significant differences in the numbers or mass of offspring produced at sites of varying radionuclide contamination was found in the present study. Significant differences in the body mass of ovigerous females were found, suggesting different timing of reproduction between localities. However, this was not related to radiation dose rates or any of the measured potential confounding factors. No support for these hypotheses were found within the data.

These findings are different from the available literature regarding the effects of chronic radiation exposure on the reproduction of natural populations (see Sect 4.1.1). These studies unequivocally suggest effects on reproduction on a range of non-human biota, many at lower doses than calculated in the present study. Though differences in radiation tolerance across phylogenetic groups may explain some of the observed differences, laboratory studies of crustaceans have also demonstrated effects at doses below those in the present study. For example, in a multigenerational experiment in *Daphnia magna*, Parisot et al., (2015) recorded effects such as a reduction in fecundity and delayed reproduction at doses of 7 µGy/hr. Two of the sampling sites in the present study exceeded these dose rates (Yanovsky Crawl & Glubokoye Lake) with no discernible effects on reproduction. As highlighted in Chapter 1, the available radioecology data suggests that organisms in the field are significantly more sensitive than those exposed to radiation under controlled conditions (Garnier-Laplace et al., 2013). In contrast, the results of this study suggest that organisms exposed under laboratory conditions may be more sensitive to chronic radiation exposure. The possible drivers for this discrepancy will be discussed further in Chapter 6.

One explanation for the differences between studies is the possibility for adaptation of *A. aquaticus* at Chernobyl. The available evidence for adaptation of non-human organisms to radiation at Chernobyl is limited at present (see Møller & Mousseau, 2016 for review), though some studies in plants have suggested adaptation in areas of higher contamination (e.g. Danchenko et al., 2009; Kovalchuk et al., 2004). Aquatic invertebrates are known to be able to respond to pollutant stress in as few as seven generations (see Sect 3.4 for further discussion), highlighting the potential of adaptation as a mechanism for the lack of observed effects in the present study. Furthermore, a number of studies have shown elevated tolerance to pollution in *Asellus* populations inhabiting contaminated environments. Brown (1976) studied populations of the closely related *Asellus meridianus* inhabiting rivers receiving mine drainage for approximately 200 years. Individuals collected from chronically contaminated sites showed elevated tolerance to both copper and lead following controlled exposures. Tolerance was found to persist in F_2 generation individuals cultured in the laboratory, suggesting a genetic basis for the tolerance. It is therefore possible that Chernobyl populations of *Asellus* may have responded to chronic radiation exposure by increased tolerance to radiation, allowing for normal reproduction.

The metabolic cost theory predicts that stress causes alterations to energy budgets resulting in detrimental effects on growth and reproduction (Calow & Sibly, 1990). This has been demonstrated for *A. aquaticus* inhabiting polluted environments (e.g. Maltby, 1991). Laboratory studies of daphnids exposed to radiation have also demonstrated a metabolic cost of internal alpha radiation exposure which was associated with a reduction in investment in offspring (Alonzo et al., 2006, 2008a). In the present study, radionuclide contamination did not impact the number or weight of eggs, suggesting no effects on reproductive investment. Furthermore, the previous chapter demonstrated no effects of chronic radiation exposure on the developmental stability of *A. aquaticus*. The lack of effect on reproduction and development in the present study may suggest that metabolic resources are being diverted from other processes in order to meet the physiological costs of radiation exposure (Jones & Hopkin, 1996). Therefore, other biological processes such as growth and respiration rate that were not monitored in the present study may have been affected. Similarly, alterations to metabolic requirements may lead to changes in feeding behaviour. Nascimento & Bradshaw (2016) demonstrated reduced grazing activity in *Daphnia magna* exposed to acute gamma radiation from ^{137}Cs. Given the importance of feeding in *A. aquaticus* on leaf litter decomposition and therefore nutrient cycling in freshwater ecosystems (Graça et al., 1994), future studies should adopt a multi-endpoint approach and metabolomic techniques to gain a broader understanding of the effects of radiation on energy allocation and biological effects.

In the present study, significant differences in maternal weight were observed suggesting different timing of reproduction between lakes of varying contamination. However, this was not found to be related to radiation dose rate or any of the other measured potential confounding factors. In *A. aquaticus,* duration of both the breeding period and embryonic development is related to temperature and the number of degree days above a minimum temperature (Anderson, 1969; Murphy & Learner, 1982; Økland, 1978). Similarly, studies have suggested a role of photoperiod and food availability in governing reproductive patterns in *A. aquaticus* (Tadini & Valentino, 1969). In the present study, sampling was conducted once per year at each locality over two sampling years. Analysis of additional environmental conditions throughout the year such as food availability and the timing of spring would allow for greater understanding of the factors driving *A. aquaticus* reproduction within these lakes and may explain the lack of a relationship between environmental parameters and *Asellus* reproduction. However, sampling throughout the year was not possible owing to logistical and permitting restrictions. In the majority of reported cases *A. aquaticus* individuals reproduce only once during their lifespan and are considered semelparous (Chambers, 1977; Murphy & Learner, 1982; Steel, 1961) though multiple broods have been recorded (e.g. Maltby, 1991). Therefore, sampling once per year is adequate to gain an understanding of typical reproductive output in *A. aquaticus*.

4.5 Conclusions

In conclusion, the present study did not observe any significant association of reproductive endpoints in crustaceans with radiation. This suggests either that there are no such effects in current *Asellus* populations, or that these effects are so subtle to be undetectable in the natural environment given other environmental influences on *Asellus* reproduction. This study represents the first assessment of reproduction in an aquatic invertebrate population chronically exposed to radiation in the natural environment. The findings of the present study will aid in our understanding of the long-term impacts of radiation exposure at higher ecological levels and support the management and monitoring of radioactively contaminated environments.

Chapter 5

Genetic Diversity along a Gradient of Radionuclide Contamination in *Asellus aquaticus* at Chernobyl

5.1 Introduction

5.1.1 Effects of Pollution on Genetic Diversity

Studies of the impacts of contaminants on genetic diversity have increased in recent years owing to the advent of affordable DNA sequencing services and the recognised importance of maintaining genetic diversity in the conservation of wildlife populations (e.g. Frankham et al., 2014; Giska et al., 2015; Rumisha et al. 2017). Genetic diversity measures are of particular importance as many commonly used indicators of pollutant exposure adequately reflect transient stress, but do not account for pollution-induced alterations to the gene pool and evolutionary processes such as genetic drift and natural selection which may be retained over long timescales (Anderson et al., 1994; Bickham et al., 2000). Persistent reductions in genetic diversity within a population may have significant implications such as a reduction in adaptive potential and increased extinction probability (McMillan et al., 2006). In seagrass for example, a number of studies have demonstrated positive correlations between genetic diversity and resilience/adaptability to environmental changes (Ehlers et al., 2008; Reynolds et al., 2012). An elevated genetic diversity associated with increased mutational load may also be detrimental to populations. Though mutations are fundamental to the evolution and adaptability of populations, the majority of mutations are deleterious to fitness (Bickham et al., 2000). Analysis of genetic diversity therefore represents a fundamental component of long-term ecological risk assessment in contaminated environments.

There are two broad ways by which pollutant exposure may impact genetic diversity within a population. Firstly, if the contaminant is mutagenic at sub-lethal concentrations then exposure of individuals would lead to new alleles (a variant form of a gene) and genotypes within a population (Belfiore & Anderson, 2001). In absence of mutagenicity, mutation rates and recombination have also been shown to increase following stress (Hoffmann & Parsons, 1991). Such mutations may be adaptive and would be enhanced owing to contaminant-driven directional selection (Taddei et al., 1997), leading to increased genetic diversity within a population. Conversely, a decrease in genetic variability may be observed if a population bottleneck occurs. Bottleneck events occur when the size of a population is reduced due to contaminant exposure, leading to a small subset of genotypes available for recovery and expansion (van Straalen & Timmermans, 2002). This would lead to reduced genetic diversity due to the direct removal of genotypes, known as ‘genetic erosion’.

Studies of natural populations inhabiting contaminated environments have demonstrated both responses. For example, significantly lower genetic diversity was recorded in populations of the tiger prawn, *Penaeus monodon*, inhabiting areas chronically contaminated with trace metals (Rumisha et al., 2017). A significant negative correlation between trace metal concentrations and levels of genetic diversity was recorded, supporting the genetic erosion hypothesis. Conversely, increased genetic diversity of the perennial plant, *Viola tricolor*, growing in soils contaminated with heavy metals was observed by Słomka et al., (2011). The authors attributed this to microevolutionary processes selecting for more tolerant genotypes in contaminated areas.

5.1.2 Effects of Radiation on Genetic Diversity in Natural Populations

Ionising radiation has been demonstrated to impact genetic diversity in a range of organisms, with most studies reporting an increased genetic diversity in populations inhabiting radioactively contaminated environments. For example, Theodorakis & Shugart (1998) demonstrated higher levels of genetic diversity in populations of mosquito fish (*Gambusia affinis*) inhabiting two ponds contaminated with radionuclides as compared to two reference populations. The authors suggested that these differences may be owing to contaminant-driven selection induced by radionuclide exposure. At Chernobyl, an increase in genetic diversity has been recorded in populations of plants (Tsyusko et al., 2006; Volkova et al., 2017), rodents (Baker et al., 2017; Matson et al., 2000), and elevated mutation rates in birds (Ellegren et al., 1997). However, these results are not ubiquitous with a number of studies recording no effect of Chernobyl-derived radiation on genetic variation (e.g. DeWoody, 1999; Livshits et al., 2001). In a meta-analysis of 45 studies spanning thirty species, Møller & Mousseau (2015) concluded that the available evidence suggested strong effects of Chernobyl-derived radiation on mutation rates. No studies have documented a reduction in levels of genetic diversity in organisms at Chernobyl.

To the author’s knowledge, only a single study of genetic diversity in an aquatic population exposed to radiation exists. This study was conducted in ponds receiving radioactive waste from the US Department of Energy’s Oak Ridge facility and the results of the study are summarised in Theodorakis & Shugart (1998). No study has focused on aquatic biota in Chernobyl-impacted lakes. This represents a significant knowledge gap which the present study aims to address.

5.1.3 Genetic Markers & Genotyping-by-Sequencing Methods

Molecular markers are the primary method by which genetic variation at a locus (a given location on a chromosome or region of genomic DNA) is measured in studies of gene diversity. A vast array of different markers have been employed over the years, owing to continuous advancement in sequencing technology and the affordability of such analyses (Schlötterer, 2004). Many of the studies of IR-induced genetic changes have employed techniques such as restriction fragment length polymorphisms (RFLPs, e.g. Theodorakis & Shugart, 1998), amplified fragment length polymorphisms (AFLPs, e.g. Volkova et al., 2017) or microsatellites (e.g. Ellegren et al., 1997). Recent studies have shown that genome-wide analysis of single nucleotide polymorphisms (SNPs) provide less biased measures of genetic diversity as compared to more traditional techniques such as microsatellites (Fischer et al., 2017). SNPs are single base pairs along a DNA sequence that vary between individuals (Freeland et al., 2011). Genetic diversity analyses using SNPs have gained popularity in recent years (see Schlötterer, 2004) and offer a range of advantages over techniques such as RFLP, AFLP and mitochondrial DNA analyses. For example, SNPs are more widely abundant across the genome as compared to microsatellites and RFLP-based techniques, and are more reproducible (Davey et al., 2011; Schlötterer, 2004).

Genotyping-by-sequencing (GBS) is a next generation sequencing (NGS) technique that focuses on a reduced representation of the genome to analyse a vast number of genetic markers (Narum et al., 2013). GBS methods offer a number of advantages in assessing population genetics and gene diversity. By screening for a large number of polymorphisms throughout the genome, markers can be analysed that are subject to the full range of evolutionary histories, e.g. natural selection, recombination and mutation (Narum et al., 2013). Previous methods utilising lower numbers of genetic markers (e.g. AFLP, RFLP, microsatellites) have reduced ability to make inferences regarding adaptive evolution. GBS methods involve the use of a restriction enzyme (RE, an enzyme which recognises particular base sequences of DNA and makes double standed cuts) to reduce the complexity of the genome and subsequent sequencing of the ends of the generated fragments (Elshire et al., 2011). A more detailed description of GBS methods will be presented in Section 5.2.2. GBS has been applied to a wide range of both model and non-model organisms and has been demonstrated to be a useful technique for population genomic analyses even where reference genomes are not available (e.g. White et al., 2013).

5.1.4 Aims & Hypotheses

At present, a data gap exists regarding the effects of chronic radiation exposure on the population genetics of aquatic organisms at Chernobyl. Furthermore, no studies to date have considered the effects of anthropogenic radionuclides on genetic diversity in crustaceans. The present study aimed to address these data gaps by employing GBS methods to determine the effects of chronic, low-level radiation exposure on *A. aquaticus* at Chernobyl over spatial and temporal scales. Samples collected closer to the time of the accident (2004/2005) were analysed to determine changes in genetic diversity over time. Based on the number of studies documenting an increase in genetic diversity in Chernobyl populations (see Sect 5.12), it was hypothesised that:

- Genetic differentiation between populations would be influenced by dose rate within habitats of *A. aquaticus*
- Populations of *A. aquaticus* would display increased genetic diversity along a gradient of radionuclide contamination at Chernobyl.

Owing to greater dose rates closer to the time of the accident and a shorter temporal scale for adaptation, it was further hypothesised that:

- Individuals analysed from 2004/2005 would display elevated genetic diversity relative to samples collected in 2015.

5.2 Materials & Methods

5.2.1 Field Sampling & Collection of A. aquaticus

A. aquaticus was collected in May and June of 2015 and 2016 following the protocol outlined in Section 3.2.1. In addition, samples were collected from two of the contaminated lakes (Glubokoye and Svatoye #3) in 2004 by colleagues at National Academy of Sciences, Belarus, following the same protocol (see Chapter 3 for further description of sampling sites).

5.2.2 DNA Extraction & Restriction Enzyme Digests for Quality Control

DNA was extracted using a QIAGEN Blood & Tissue Kit (QIAGEN, Germany) following some amendments to the manufacturer protocol to maximise DNA yield. *A. aquaticus* samples were first weighed using a Kern ABT 120-5DM analytical balance, and samples < 5 mg were pooled for subsequent DNA extraction. Male individuals were selectively used for extraction to reduce variability associated with sex-specific genes. Individuals were first homogenised using a micropestle in 180 µl of Buffer ATL in a 1.5 ml microcentrifuge tube. Following addition of 40 µL of Proteinase K, samples were vortexed and incubated at 56 °C for 16 hours to allow for lysis to occur. Samples were centrifuged at 20,000 g for 5 minutes and the supernatant transferred to a new microcentrifuge tube. This step was performed to remove the non-lysed, largely chitinous tissue as this may impact the quality of DNA for downstream applications (Verovnik et al., 2003). Following thorough mixing, 200 µl of Buffer AL was added to the sample followed immediately by 200 µl of 96% Ethanol (Sigma-Aldrich, UK). Samples were mixed and transferred to a DNeasy spin column within a 2 ml collection tube and centrifuged at 6000g for 1 minute. The flow-through was discarded and the spin column was placed within a fresh collection tube. Washes were performed by addition of 500 µl of Buffers AW1 and AW2 and centrifugation. Following the second wash, samples were centrifuged at 20,000 g for 3 minutes to dry the DNeasy membrane. After addition of the spin column to a clean microcentrifuge tube, 102 µL of Buffer AE preheated to 50°C was added directly onto the membrane. Samples were incubated at room temperature for 10 minutes, and elution performed by centrifugation at 6000g for 1 minute.

The quantity and quality of the DNA was first analysed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, UK), as DNA quality is a key determinant of the outcome of GBS studies (Peterson et al., 2014). The ratio of absorbance at 260/280 nm and 260/230 nm were analysed to ensure DNA with optimum purity was used for downstream applications and to determine the concentration of DNA. Nucleic acids and proteins have absorbance maxima at 260 and 280 nm respectively. The ratio between absorbances at these wavelengths is a commonly used measure of purity of extracted DNA, with a 260/280 ratio of ~ 1.8 generally accepted as pure. Expected values for the 260/230 ratio, a measure of other contaminants, is typically higher with values of 2.0 – 2.2 acceptable.

For GBS analysis, a volume of 30 µL of high quality DNA at a concentration of between 30 – 100 ng/µL was required. Any extracted DNA with a concentration of greater than 100 ng/µL was diluted with Buffer AE. Restriction enzyme digests and visualisation by agarose gel electrophoresis were performed on 10% of DNA samples to allow for assessment of DNA quality by collaborators at the Genomic Diversity Facility, Cornell University. Digests were performed with the non-methylation sensitive restrictive enzyme, *Hind*III (Thermo

Fisher Scientific, UK) according to the manufacturer's protocol. A volume of 500 ng of DNA was added to 16 µl of nuclease-free water, 2 µl of 10 x Buffer R and 0.5 µl of *Hind*III (10U/µl) and mixed thoroughly. Following incubation at 37°C for at least 3 hours (1-16 hours recommended), *Hind*III was thermally inactivated by incubation at 80 °C for 20 minutes.

The results were visualised by agarose gel electrophoresis. Samples were ran on a 1% agarose gel in 1 x TAE (National Diagnostics, UK) along with 500 ng of *HindIII* ladder (Thermo Fisher Scientific, UK) at a voltage of 7 V/cm for 3 hours. The gels were prepared by dissolving 1g of Agarose (Thermo Fisher Scientific) in 100 mL 1 x TAE and microwaved for approximately 1 minute to dissolve agarose. After cooling, gels were poured into a casting tray with a plastic comb and allowed to cool for between 20 – 30 minutes. Following cooling, samples were mixed with 6 x loading dye (Thermo Fisher Scientific) and loaded into lanes. After electrophoresis under the conditions mentioned above, gels were stained using 1 x SYBR Gold (Molecular Probes, UK) in TAE and visualised using a VWR Smart Gel Documentation System (VWR, US). An example of the results is shown in Figure 5.1. The band visible in the uncut samples represent high molecular weight *A. aquaticus* genomic DNA.

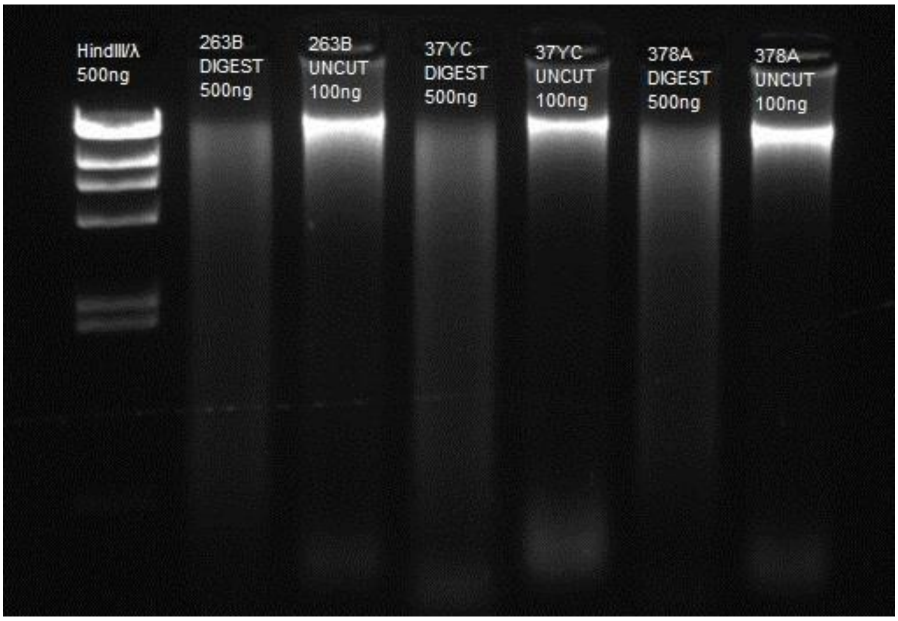

Figure 5.1 – Example of a restriction enzyme (*Hind*III) digest in *A. aquaticus* genomic DNA visualised on a 1% Agarose Gel and stained with SYBR Gold. Numbers represent individual samples and amounts of DNA.

Following confirmation of acceptable DNA quality for GBS analyses, samples were arrayed on to a 96-well fully skirted PCR plate (Applied Biosystems, US) and sealed using PCR caps (Thermo Fisher Scientific). Samples were arrayed randomly with one blank as a negative control and shipped to the Genomic Diversity Facility at Cornell University's Institute of Biotechnology at ambient temperature. The numbers of samples analysed per site are shown below in Table 5.1.

Table 5.1 – Number of samples analysed per site for Genotyping-by-sequencing.

Site		Number of Samples Sequenced
Gorova		13
Dvorische		12
Stoyecheye		12
Svatoye #3	2015	12
	2004	10
Yanovsky Crawl		12
Glubokoye	2015	13
	2004	11

5.2.3 Genotyping-by-Sequencing

All DNA sequencing and initial bioinformatics analysis were performed at the Genomic Diversity Facility, Cornell University, New York. Preliminary optimisation was conducted on DNA samples to determine the most appropriate RE to generate libraries for GBS analysis. The results of the optimisations are shown below in Figure 5.2. Selection of the appropriate RE is one of the key considerations of GBS studies (Pan et al., 2015). Owing to the greater range of fragment sizes generated (see Figure 5.2), PstI was selected as the appropriate RE for the present study. A schematic for the basic workflow for GBS studies is shown in Figure 5.3. The specific methods for GBS used at the Genomic Diversity Facility at Cornell University have been described and published by Elshire et al., (2011) and are summarised below.

Each DNA sample within the 96-well plate was first digested using PstI. Fragmented DNA was then ligated to both a barcoded and a common adaptor (a short oligonucleotide that can be ligated to the ends of DNA). A barcode refers to a short sequence used to identify a given adaptor, as samples are ultimately mixed and sequenced together. Each well of the plate contained genomic DNA from a different individual and a unique barcoded adaptor molecule. Following ligation of the adaptor, samples were pooled within a single Eppendorf tube and purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, USA). The library was then subjected to the polymerase chain reaction (PCR) using primers that corresponded to the ligated adaptors. The PCR step serves two functions; size selection, as the PCR preferentially amplifies fragments of the most suitable length for sequencing, and the addition of a length of sequence to the primers which is bound to the Illumina flow cell during sequencing (White et al., 2013). Following the PCR, samples were purified once again in the manner described above. After appropriate dilution of samples, sequencing was performed using the Illumina Hi-Seq 2000 at the Cornell University Core Laboratories Centre.

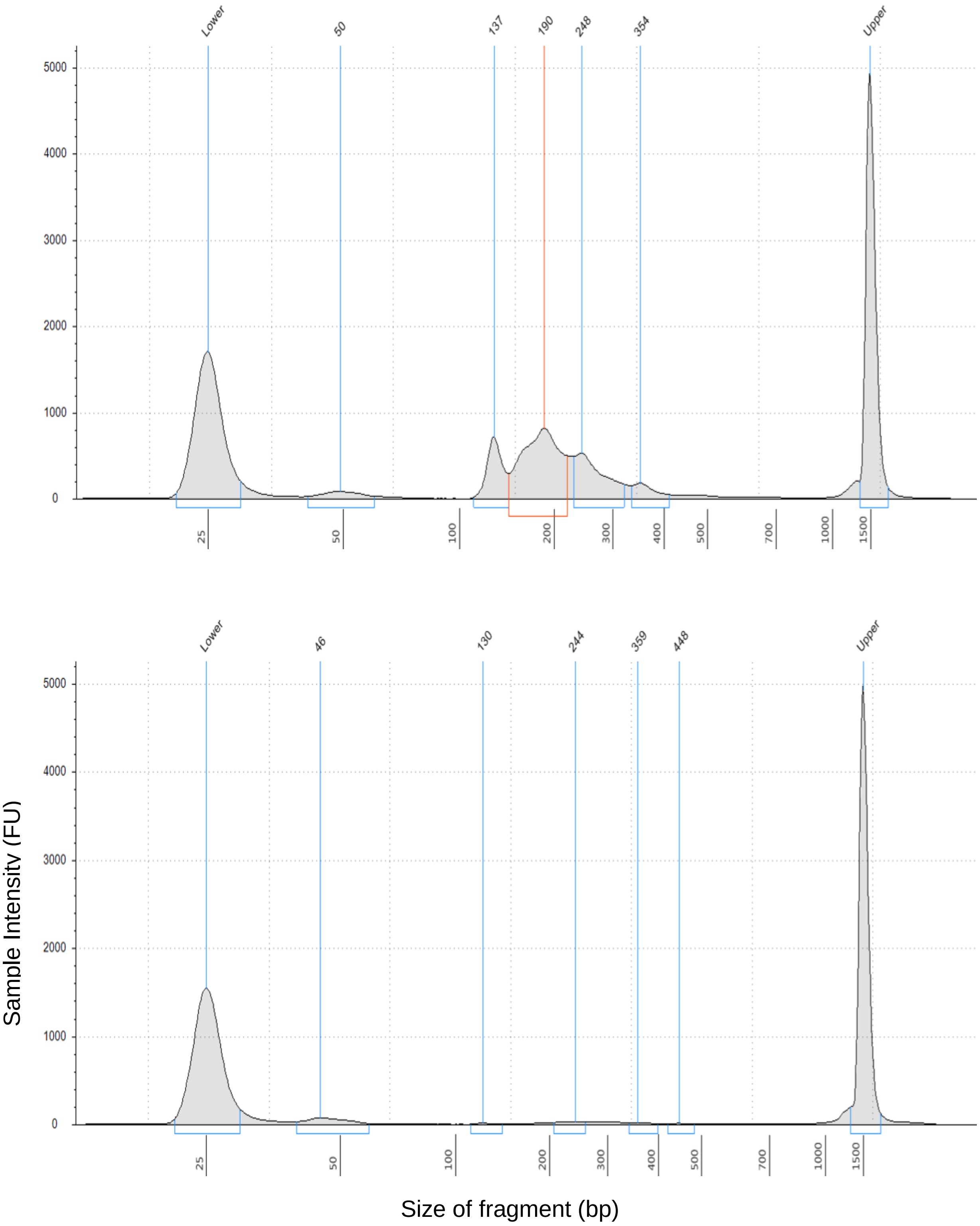

Figure 5.2 – Restriction enzyme optimizations in *A. aquaticus* genomic DNA. Results are fragment size analyses of the distribution of GBS libraries prepared using PstI (top) and SbfI/BfaI (bottom) double digests.

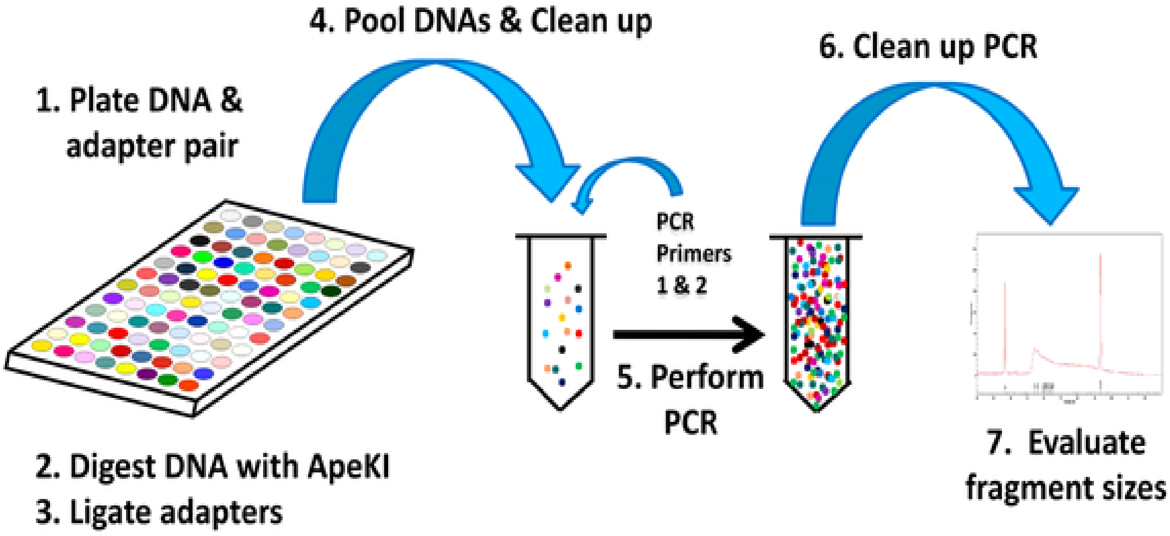

Figure 5.3 – Basic workflow for performing Genotyping-by-sequencing. Image from Elshire et al., (2011).

5.2.4 Bioinformatics & SNP Calling

Bioinformatics analysis was conducted by collaborators at the Genomic Diversity Facility using the UNEAK (Universal Network-Enabled Analysis Kit, Lu et al., 2013) pipeline and the TASSEL software version v 3.0 (Trait Analysis by aSSociation, Evolution and Linkage, Bradbury et al., 2007). The UNEAK pipeline was developed to overcome the issues associated with SNP discovery in absence of a reference genome (Lu et al., 2013). The UNEAK pipeline works by first trimming all sequence reads to 64 base pairs (bp). The trimmed parts can be ignored for analysis as sequencing errors (e.g. erroneous base calls) are enriched in these areas. Reads of 64bp that are identical are then identified as tags, and pairwise alignment identifies pairs of tags with a single base pair mismatch. These are known as candidate SNPs. Such tags are aligned to form a network and these networks are pruned to remove putative sequencing errors (low frequency alleles). Pruning was conducted according to an error rate threshold parameter of 0.01%. Only reciprocal tag pairs are used for subsequent SNP calling, meaning only biallelic loci were studied. If two alleles occur at a given locus within a population, this is said to be biallelic or diallelic (Elston et al., 2012).

VCF tools (v0.1.12a) was then used to calculate depth and missingness statistics for generated SNP files. Heterozygosity and minor allele frequencies were also calculated using VCF tools. Multi-dimensional scaling (MDS) was performed on genome-wide SNP data using both VCF tools and PLINK (v1.07), a software package used for genome association analysis.

5.2.5 Statistical Analyses

The R package SNPrelate (Zheng et al., 2012) was used to calculate additional diversity statistics and to create a principal component analysis (PCA) plot of genotypes from filtered SNP data. A matrix of Fst values were calculated according to the method of Weir & Cockerham (1984). Fst values are a measure of genetic differentiation between populations and are fundamental in studies of population genetics. A larger value indicates greater genetic differentiation between populations, with values greater than 0.15 generally considered to indicate significant differentiation (Frankham et al., 2002). Other measures of genetic diversity such as Tajima’s D, nucleotide diversity and expected heterozygosity were calculated using the R Package PopGenome (Pfeifer et al., 2014). Tajima’s D statistic (Tajima, 1989) is used to distinguish whether a given DNA sequence has evolved randomly, or has been subjected to a non-random process e.g. directional selection. In order to test for the presence of Isolation-by-distance (IBD), the linear relationship between geographical and genetic distance measured by Fst, a Mantel test was used via the R package vegan (Oksanen et al., 2013). IBD is the process by which geographical distance structures population genetic patterns owing to limited gene flow (Hardy & Vekemans, 1999), and is an important consideration in population genetic studies. To test for the influence of the gradient in dose rate on genetic differentiation, a partial mantel test was used based on Pearson’s product-moment correlation. This test measures the association between two matrices (e.g. genetic distance and Euclidean distance in dose rate) whilst accounting for a third potentially confounding matrix (geographical distance). The association between calculated genetic diversity measures and dose rate was calculated using Spearmans rank order correlation coefficient. Samples collected in 2015 only were used for correlations of radiation dose rate and genetic diversity measures. Differences in genetic diversity between the two sampling years were tested using two-way ANOVA with site and sampling year used as fixed factors. Power analysis was employed to determine the probability of seeing different effect sizes based on sample size using the R package “pwr” (Champely et al., 2009).

5.3 Results

5.3.1 Data Quality & Coverage

Illumina sequencing of 95 individuals on one lane yielded a total of 492 402 538 reads. Of these, 372 029 165 were classed as ‘good’ reads containing a unique barcode. Depth of coverage was on average 25.74, meaning each nucleotide has been sequenced on average 25 times. A depth of coverage > 20 times is preferred in SNP studies to redue the uncertainty associated with calling SNPs following low-coverage sequencing (e.g. 5 x depth of coverage, Nielsen et al., 2011). At lower sequencing depths, there is an increased probability that only one chromosome of a diploid individual has been sampled at a given site, which introduces significant uncertainty in SNP and genotype calling. The UNEAK pipeline identified a total of 32,321 SNPs which was trimmed to 14,463 following the data filtration steps outlined above.

5.3.2 Genetic Differentiation & Isolation-by-Distance

Genetic differentiation values (Fst) are shown in Table 5.3. A larger value represents greater genetic differentiation, whereas smaller values indicate more genetically similar populations. Greatest values were observed between Svatoye Lake and Glubokoye 2015 (0.272), suggesting these populations were the most genetically isolated from each other. Smallest values were recorded between the two sampling years within Glubokoye and Svatoye

Lakes (0.075 & 0.072 respectively), indicating the genetic similarity of these populations over time. A highly significant relationship between geographical distance in kilometres and genetic differentiation was recorded (Mantel Test, r = 0.867, p < 0.01), confirming the Isolation-by-distance hypothesis. The relationship between geographical and genetic distance between sites is shown in Figure 5.4.

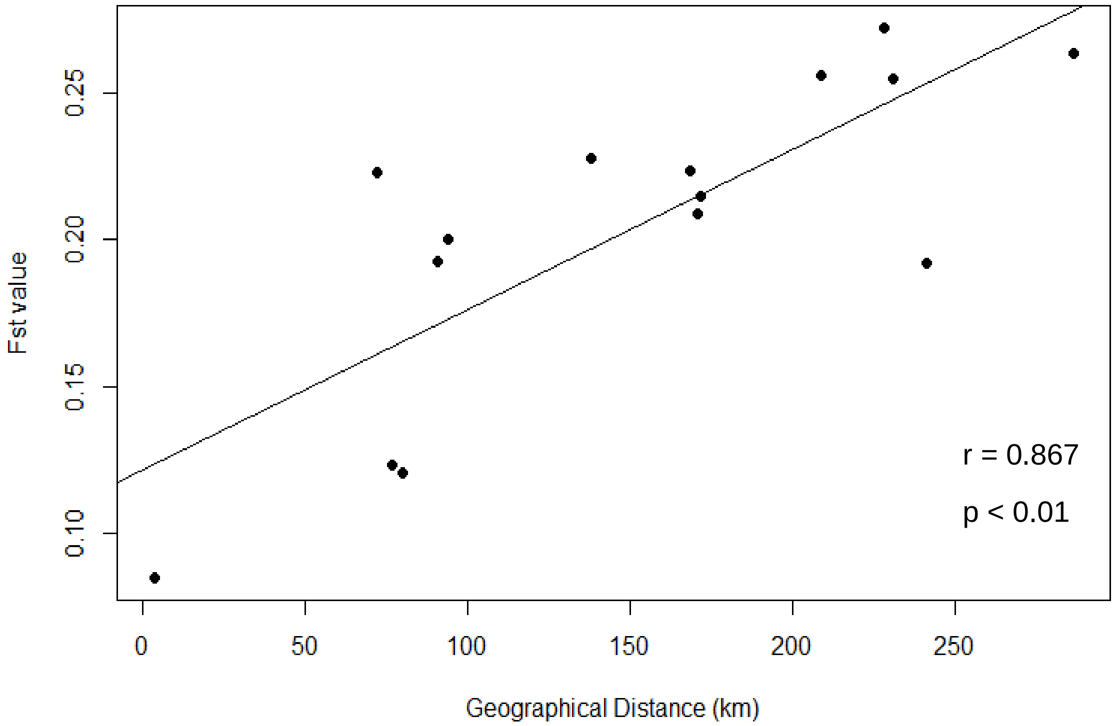

Figure 5.4. – Results of the Mantel test showing the relationship between geographical distance and genetic differentiation (Fst) between six sites of varying contamination in *Asellus aquaticus.*

Conversely, no significant effect of the gradient in dose rate (Euclidean distance) on genetic differentiation was recorded when accounting for geographical distance (see Figure 5.5, Partial Mantel Test, r = 0.251, p > 0.05). This indicates that dose rate did not influence genetic differentiation amongst populations at Chernobyl. However, geographical distance was found to be a driver of genetic differentiation. A PCA plot of genotypes based on genome-wide filtered SNP data is shown in Figure 5.6. The PCA analysis clearly separated populations.

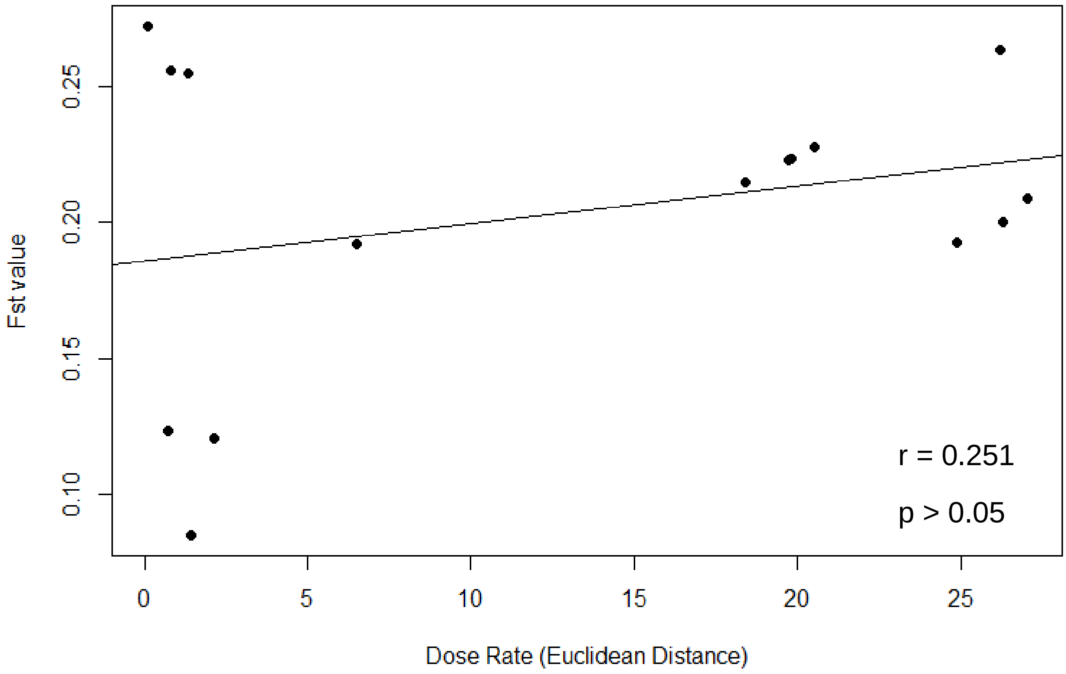

Figure 5.5 – Results of the Mantel test showing the relationship between the gradient in dose rate (as Euclidean distance) and genetic differentiation (Fst) between six sites of varying contamination in *Asellus aquaticus* at Chernobyl.

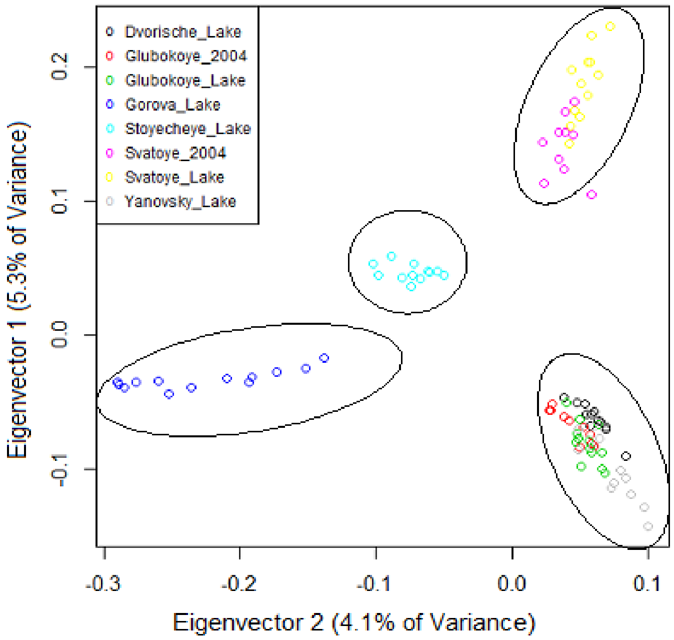

Figure 5.6 – Principal component analysis of genotypes based on genome-wide SNP data of *A. aquaticus* collected from six lakes along a gradient of radionuclide contamination at Chernobyl.

5.3.3 Genetic Diversity in Relation to Radionuclide Contamination

Summaries of the calculated genetic diversity statistics for each population are shown in Table 5.2. Expected heterozygosity (H_e) is the most commonly used measure of gene diversity, as it accounts for differences in allele richness and evenness and is therefore less subject to bias than other measures (Nei, 1973). Lowest values for H_e were recorded at the two sites sampled in 2004, being 0.221 and 0.228 for Glubokoye and Svatoye 2004 respectively. No significant relationship between H_e and total dose rate was recorded (see Figure 5.7, n = 74, Spearmans rho = -.110, $p > 0.05$). Nucleotide diversity (π), a measure of polymorphism within the population (calculated as average number of nucleotide differences per site between two DNA sequences in all possible pairs of a sample population, Wakeley, 1996), ranged from 0.0011 at Glubokoye 2004 to 0.0026 at Gorova. No significant relationship between nucleotide diversity and dose rate was recorded (Spearmans rho = -0.333, $p > 0.05$).

Similarly, no significant relationship between dose rate and observed heterozygosity (H_o, the percentage of loci heterozygous per individual) or the within population inbreeding coefficient, F (the probability that two alleles are identical by descent from a common ancestor), was recorded (Spearmans rho = -0.018, $p > 0.05$ and rho = -0.031, $p > 0.05$ respectively). Coupled with the lack of a relationship between genetic differentiation and dose rate, this provides evidence that genetic patterns in *A. aquaticus* at Chernobyl were not influenced by dose rate. Tajima's D statistic was < 0 for all populations (see Table 5.2), with the lowest value recorded at Glubokoye in 2004 (-1.367) and the greatest value recorded at Svatoye in 2004 (-0.248). A moderate negative relationship between Tajima's D values and dose rate was recorded, though this was non-significant (Spearmans rho = -0.5, $p > 0.05$).

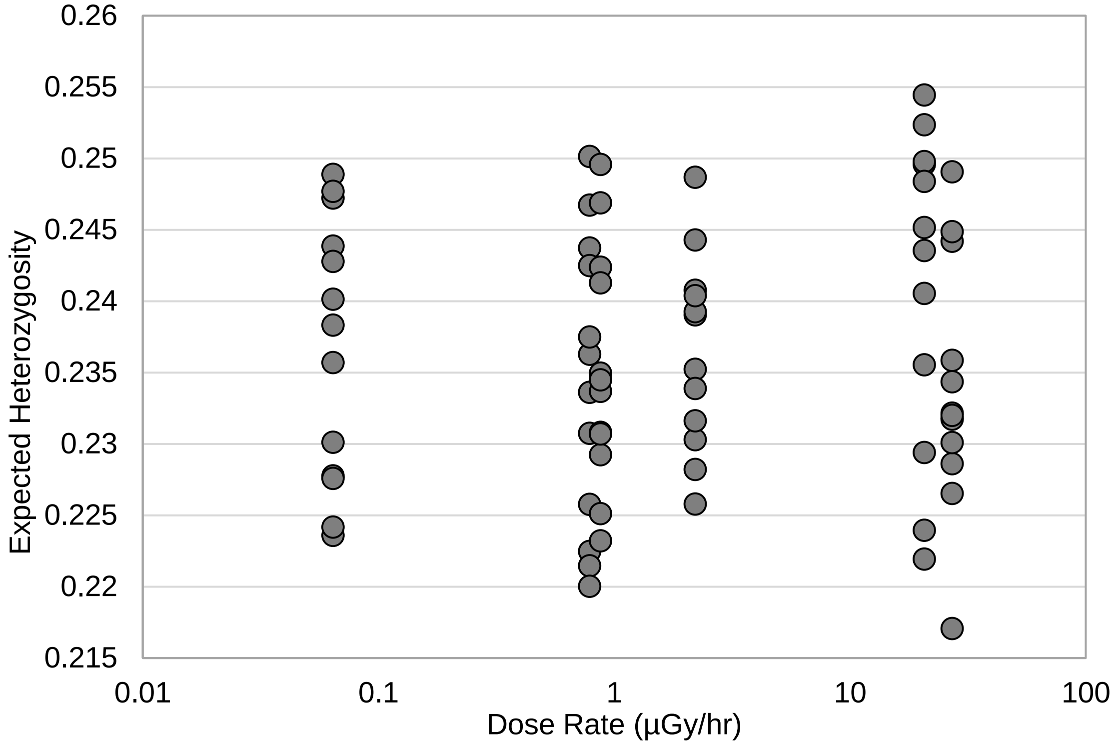

Figure 5.7 – Relationship between expected heterozygosity (gene diversity) and dose rate in *Asellus aquaticus* samples collected from six lakes along a gradient of contamination at Chernobyl.

5.3.4 Alterations to Genetic Diversity over Temporal Scales

For expected heterozygosity, H_e, significant differences were recorded between sampling periods owing to significantly lower H_e in 2004 as compared to 2015 at both Glubokoye and Svatoye 3 (ANOVA, $F_{1, 45} = 27.202$, $p < 0.05$). No significant interaction between sampling site and year was recorded (ANOVA, $F_{1, 45} = 1.177$, $p > 0.05$) indicating that, for H_e, differences between years were not dependent on sampling site. For H_o and the inbreeding coefficient, F, no significant differences between year or site were recorded (ANOVA, $p > 0.05$). However, significant interaction terms (ANOVA, $p < 0.05$) indicated that differences in H_o and F between years were dependent on site. For H_o, lower values were recorded in 2004 relative to 2015 at Svatoye, with the inverse relationship observed at Glubokoye (see Figure 5.8). For F, greatest values were observed in 2004 as compared to 2015 at Svatoye and the inverse relationship 2015 at Glubokoye. Other genetic diversity measures (Nucleotide diversity and Tajima's D) are calculated at the population level, therefore comparisons between sampling years were not valid. Given the different responses in the genetic diversity measures over time, e.g. lower values for H_e in 2004 but higher values for H_o and F at Glubokoye, it is concluded that no overall significant increase in gene diversity over temporal scales was recorded.

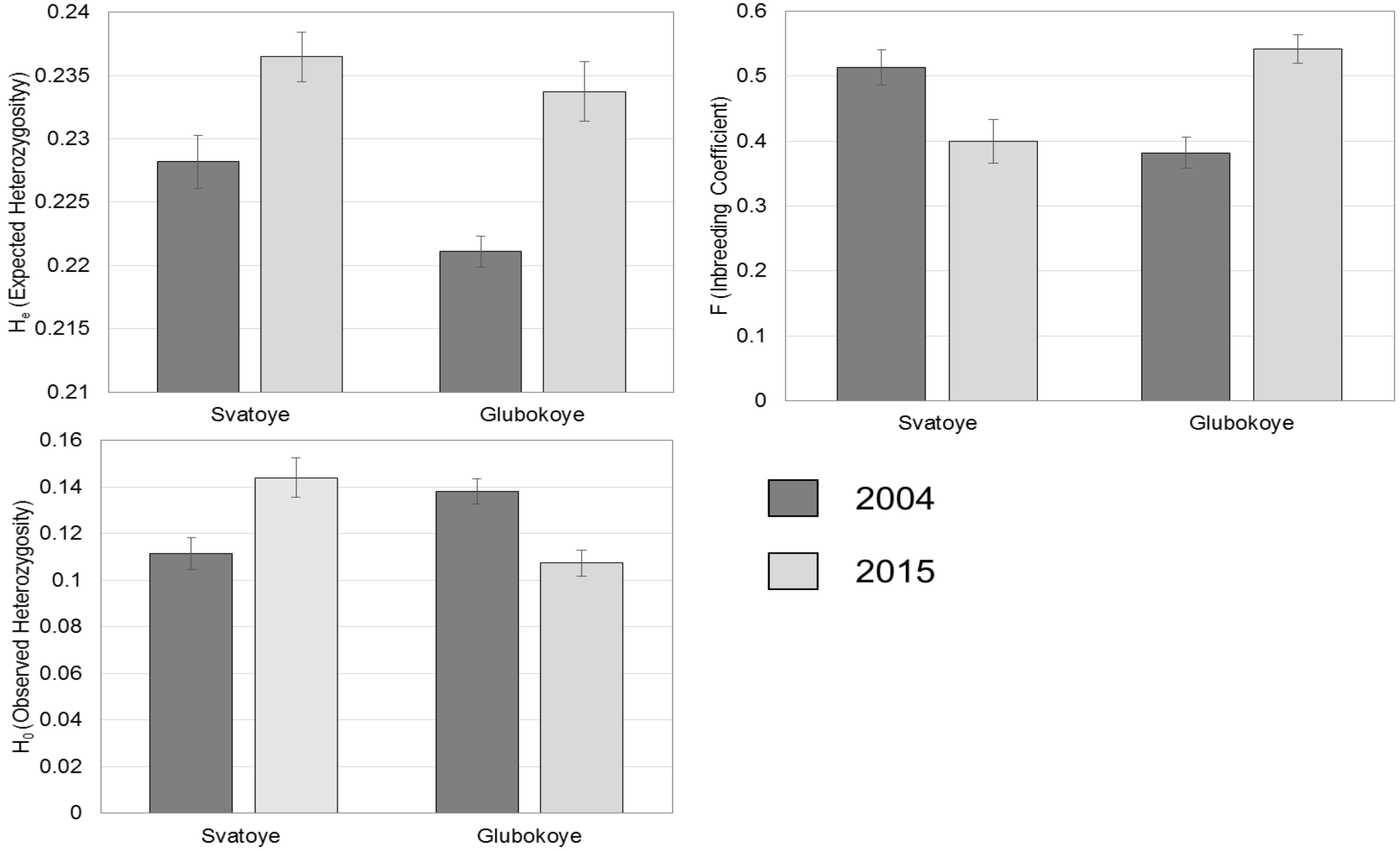

Figure 5.8 – Genetic diversity parameters (H_e, H_0 and F) in *Asellus aquaticus* at two contaminated lakes in Chernobyl in 2004 and 2015. Error bars are ± standard error of the mean.

Table 5.2 – Calculated genetic diversity statistics for each population based on genome-wide SNP data.

	Expected Heterozygosity	Observed Heterozygosity	F (Inbreeding Coefficient)	Nucleotide Diversity (π)	Tajima's D
Dvorische	0.234263517	0.123434729	0.47537	0.001779881	-1.074
Glubokoye 2004	0.22109079	0.137941992	0.376792727	0.001143985	-1.367
Glubokoye 2015	0.233732111	0.10738999	0.541696154	0.002438229	-1.099
Gorova	0.236766239	0.130591783	0.449427692	0.002669943	-0.659
Stoyecheye	0.235211161	0.127497693	0.457923636	0.002215685	-0.671
Svatoye 2004	0.228176951	0.111307505	0.513253	0.002321633	-0.248
Svatoye 2015	0.236464769	0.143921815	0.393421667	0.001649979	-0.612
Yanovsky Crawl	0.24123347	0.145488569	0.4021825	0.002023974	-0.984

Table 5.3. – Genetic differentiation (Fst) values calculated following the methods of Weir & Cockerham using the SNPrelate package in R Studio. See section 5.2.5 for discussion of the importance of Fst values in population genetic studies.

	Dvorische	Glubokoye 2015	Glubokoye 2004	Svatoye Lake	Svatoye 2004	Yanovsky Crawl	Stoyecheye	Gorova
Dvorische								
Glubokoye 2015	0.1235103							
Glubokoye 2004	0.1245895	0.07482516						
Svatoye Lake	0.255504	0.2719511	0.2684039					
Svatoye 2004	0.2550496	0.2678087	0.2641963	0.07178065				
Yanovsky Crawl	0.1206123	0.08523209	0.08999583	0.254953	0.2541075			
Stoyecheye	0.2278073	0.2233065	0.2265027	0.223041	0.224619	0.21475		
Gorova	0.2086033	0.1999745	0.2019091	0.263433	0.2570319	0.1927364	0.1918563	

5.4 Discussion

The present study hypothesised that genetic differentiation of *A. aquaticus* (measured here using Fst) would be influenced by radiation dose rate at Chernobyl. It was further predicted that genetic diversity would be positively related to the degree of radionuclide contamination and that samples collected in 2004 would show elevated genetic diversity as compared to 2015 samples. No significant relationship between genetic differentiation or genetic diversity and dose rate was recorded, leading to rejection of these hypotheses. Though alterations to genetic diversity were observed between the sampling years, no clear increase in genetic diversity was recorded at two contaminated sites in 2004 as compared to 2015, leading to rejection of this hypothesis. A strong significant relationship between geographical distance and genetic differentiation confirmed the Isolation-by-distance model. Calculated genetic diversity measures for *A. aquaticus* in the present study were within the range of other areas of Europe as recorded by Verovnik et al., (2005). The authors recorded mean values for nucleotide diversity, π, in populations of *A. aquaticus* across central and Eastern Europe of 0.00189 ± 3.79E-06 using mitochondrial DNA methods. Values obtained within the present study were broadly similar, ranging from 0.00114 – 0.00267 (see Table 5.2). The finding that genetic diversity in *A. aquaticus* populations chronically exposed to radiation were within the range of reference localities and other areas within Europe (Verovnik et al., 2005) is important in predicting the long term success of aquatic invertebrate populations within radioactively contaminated environments.

The suggestion of IBD in the present study is in disagreement with previous work conducted by Verovnik et al., (2005) who studied the mitochondrial DNA and 28s rDNA sequences of *A. aquaticus* at a continental scale and found no evidence of IBD. The present study focused largely on closed lake systems with limited connectivity, which is fundamental to structuring *A. aquaticus* populations given known limitations on dispersal capacity (e.g. Sworobowicz et al., 2015; Verovnik et al., 2005). This may explain the presence of IBD in the present study, as it is assumed gene flow (the movement of alleles between previously separate populations) would not have occurred between enclosed, isolated populations. Methodological differences in the calculation of genetic differentiation and techniques used to assess genetic structure may further explain the differences between studies. For example, studies have shown significant variation in the ability of genetic markers e.g. microsatellite or SNP based techniques to distinguish genetic differentiation of populations (e.g. Bradbury et al., 2015).

Gene flow between populations is often thought to mask the effects of pollutants on genetic patterns, even in species with known limited dispersal capacity (e.g. Giska et al., 2015; Theodorakis et al., 2001). However, the moderate to high levels of genetic differentiation observed between populations in the present study (Fst values up to 0.27, See Table 5.3) and the confirmation of IBD suggests that gene flow and homogenization of populations is likely not responsible for the lack of a relationship between genetic variables and dose rate recorded for 2015 samples. This suggests that current dose rates received by *A. aquaticus* at Chernobyl (maximum of 27.1 µGy/hr) are either insufficient to cause mutations and subsequent elevated genetic diversity, or that effects may have occurred previously but populations have recovered. These findings contradict the majority of studies which record an increase in genetic diversity in a range of non-human organisms (see Section 5.12).

One possible explanation for these differences may be that *A. aquaticus* populations have not been historically exposed to dose rates as high as those for rodents and pines that have been shown to have altered genetic diversity (e.g. Baker et al., 2017; Volkova et al., 2018). For example, many of the studies of genetic diversity in bank voles at Chernobyl have monitored populations from the red forest (e.g. Baker et al., 2017; Matson et al., 2000). The red forest refers to the highly contaminated area adjacent to the CNPP where acute doses led to mortality in a 4 km^2 zone of pines immediately following the accident (see Section 1.1.3i, Kryshev et al., 2005). Dose rates in this area remain extremely high to date, with maximum air dose rates of 200 µGy/hr recorded in some ‘hotspots’ within the forest (N.A. Beresford, pers. comm). Studies on the accumulation of radionuclides in small mammals within this area in the late 1990's suggested that chronic dose rates could be as high as 86 mGy/d for the duration of their lifecycle (Chesser et al., 2000, 2001). Similarly, many of the studies demonstrating genetic effects in scots pine are based on trees receiving doses orders of magnitude higher than the present study (e.g. Kuchma et al., 2011; Vornam et al., 2012), though effects at lower doses have also been recorded (e.g. Geras'kin & Volkova, 2014; Volkova et al., 2017). Though maximum dose rates in aquatic systems immediately following the accident were 100 – 200 mGy/d from bottom sediments, this applied to the Chernobyl cooling pond which was not sampled in the present study (Kyshev et al., 2005). These dose rates declined relatively rapidly owing to the decay of short lived radionuclides. Dose rates to benthic organisms at the most contaminated lake in the present study, Glubokoye, may not have been as high and may have been insufficient to induce discernible genetic effects. Coupled with the greater radiosensitivity of pines and small mammals as compared to aquatic invertebrates, this is proposed as the main driver for the lack of effects on genetic diversity in the present study.

5.5 Conclusions & Limitations

This study utilised the latest next generation techniques and greater than 14,000 genetic markers across the whole genome of *A. aquaticus* to provide a robust understanding of the effects of chronic radiation exposure on genetic diversity. This study is the first to consider genetic diversity of aquatic organisms at Chernobyl and significantly increases our understanding of the effects of chronic radiation exposure on population genetics. No effect of radiation dose rate on genetic variation was recorded, which is different to the majority of available literature recording increased genetic diversity and elevated mutation rates in non-human organisms at Chernobyl. This was attributed to lower received dose rates in *A. aquaticus* as compared to more radiosensitive organisms such as pines and rodents wherein clear genetic effects have been demonstrated (e.g. Baker et al., 2017; Volkova et al., 2017).

To the author's knowledge, this study is the first to apply a genome-wide SNP approach to studying genetic diversity in response to radiation exposure. The majority of available studies have used techniques such as AFLP, RFLP, microsatellites or mitochondrial DNA analyses (see Section 5.13 for further discussion). Given the advantages of genome-wide SNP discovery techniques as compared to previous approaches (Schlötterer, 2004), this method would have increased analytic power to detect a relationship between radiation dose rate and genetic diversity. For example, the sequencing depth in the present study (>20x) is known to provide accurate genotype calling and *de novo* SNP discovery even in non-model organisms without a reference genome (Andrews et al., 2016). The number of SNPs studied in the present study has been shown to provide reliable measures of genetic measures in non-model organisms. For example, Hoffman et al., (2014) found that inbreeding coefficients generated from RAD-seq analysis of 13,000 SNPS in the oldfield mouse, *Peromyscus polionotus subgriseus*, had strong concordance with known pedigree based values.

One of the limitations of the present work was the limited number of individuals sequenced per site and lack of an adequate number of samples collected at various time points. For analysis of the relationship between total dose rate and a range of gene diversity measures, 74 samples were analysed. For an 80% chance of detecting a 'medium' effect size of r = 0.3 (Cohen, 1988), 84 samples would be necessary assuming a significance level of 0.05. A much larger sample size of > 700 samples would be necessary for the same conditions for a 'small' effect size of 0.1 (Cohen, 1988). It is therefore possible that radiation may have induced a minor effect on genetic diversity in *A. aquaticus* that the present study may not have had the necessary statistical power to detect. In order to discriminate the drivers regulating genetic variation at the population level, multiple sampling years of a greater number of individuals and a robust understanding of ecological processes within sampling locations is necessary (Matson et al., 2000). Ideally, samples collected prior to the Chernobyl accident and those exposed to the highest dose rates in the immediate aftermath would be available for population genetic analysis. However, such samples were not available. Future research into the effects of chronic radiation exposure on genetic variation should aim to couple sampling over long time scales with a robust understanding of community dynamics.

Chapter 6

General Discussion

6.1 Are Laboratory Radioecology Studies Applicable to the Natural Environment?

One of the overarching aims of this PhD project was 'To establish the applicability and relevance of laboratory radioecology studies to the natural environment'. Disparities in the sensitivity of organisms to pollutants between controlled laboratory exposures and the field have been recorded in radioecology (Garnier-Laplace et al., 2013) and in ecotoxicology (Barata et al., 2000). This is problematic for understanding the risk posed by a given contaminant in the natural environment. Consequently, the use of combined laboratory and field studies was agreed as a priority area for future research by the International Union of Radioecology in 2015 (Bréchignac et al., 2016). This study adopted a combined laboratory and field approach to assessing the effects of chronic low-dose radiation on crustaceans. Organisms were collected from radionuclide contaminated areas at Chernobyl and Fukushima and controlled laboratory exposures of crustaceans were conducted in parallel.

In laboratory studies, significant effects were recorded on the male fertility of the marine amphipod, *Echinogammarus marinus* at ^{32}P doses of 1 mGy/d. This experiment was replicated and the same effect was observed. Knock-on impacts on the number of offspring produced in unexposed female *E. marinus* breeding with males exposed to lower doses of 0.1 mGy/d were also observed, though an experimental repeat would be necessary to confirm this result (see Chapter 2 for full discussion). These dose rates (equivalent to ~ 4.2 and 42 µGy/hr) are significantly lower than values wherein deleterious effects to populations of aquatic organisms are expected based on a number of international radioprotection benchmarks (e.g. ICRP, 2008). These laboratory studies provide evidence that aquatic organisms may be more susceptible to radiation exposure than previously thought. However, field studies across multiple endpoints including reproduction, developmental stability and genetic diversity revealed no effects of chronic radiation exposure in two crustacean species at comparable dose rates of up to 38.1 µGy/hr (Chapters 3, 4 and 5).

A previous study comparing terrestrial wildlife data at Chernobyl and controlled laboratory exposures found that organisms in the field were up to eight times more sensitive to radiation as compared to data derived from laboratory exposures (Garnier-Laplace et al., 2013). However the field data was derived from only eight references, some of which have been subject to criticism for failing to consider the full range of confounding factors (e.g. Beresford & Copplestone, 2011; Møller & Mousseau, 2009). The results of the present work contradicted the previous hypothesis (of greater effects in the field than the lab), finding greater sensitivity of organisms following controlled laboratory exposures as compared to those in the field. Though the present study contradicts the limited available data in the field of radioecology, many wider ecotoxicology studies have noted greater or similar sensitivity of populations exposed to pollutants in the lab as opposed to the field. For example, Schroer et al., (2004) compared toxicity of the pesticide, Λ-Cyhalothrin, to freshwater invertebrates under both lab and semi-field conditions and found laboratory derived sensitivity values generally provided conservative estimates of the potential for field effects. However, the authors (Schroer et al., 2004) reported that for some organisms including the model organisms used in the present study (*Asellus aquaticus* and *Gammarus pulex*), derived toxicity values were markedly different between laboratory and field conditions. The half maximal effective concentration (EC_{50}) value was three-fold greater in field populations of *A. aquaticus* as compared to in the lab, indicating that effects occurred at lower doses in the lab as compared to the field. Similarly, Spurgeon & Hopkin (1995) recorded toxicity of Zinc to the earthworm *Eisenia fetida* at concentrations ten times lower in artificial exposures as opposed to field populations, though bioavailability may have played a role.

In the field, organisms may be exposed to fluctuating or unfavourable environmental conditions in conjunction with toxicant exposure, as opposed to optimal conditions in the laboratory (Barata et al., 2002). Owing to the greater control over specific environmental conditions, organisms exposed under laboratory conditions may appear to be more sensitive than those under field conditions (Versteeg et al., 1999) Laboratory exposures are normally conducted on single-species over shorter time scales than typical field studies (Brix et al., 2011). For example, an exposure duration of two weeks was used for laboratory exposures in the present study as opposed to field organisms which have been subject to lifetime exposure to low-dose radiations. These differences may explain the observed discrepancy between lab and field results in the present study. For example, organisms may have acquired tolerance over long-term exposure to pollution in the field (see Discussion in Chapters 4 and 5, Fraser, 1980). Evidence suggests that elevated tolerance to pollutants is generally related to an increased genetic diversity (e.g. Bourret et al., 2008). However in Chapter 5, no effect of chronic radiation exposure on the genetic diversity of *A. aquaticus* was observed, suggesting that adaptation may not be the driver of observed differences.

There are number of further limitations associated with the comparison of laboratory and field data in the present study. Firstly, different species were used between laboratory and field experiments. Significant differences in a given organisms response to xenobiotic exposure has been recorded amongst closely related species. For example, in a study of two of the genera used for lab and field comparison in the present study, Lukančič et al., (2010) found significantly greater sensitivity of *Gammarus fossarum* to short term pesticide exposure as compared to *A. aquaticus*. Greater tolerance to stress in *Asellus* as opposed to *Gammarus* species has been further recorded by Graça et al., (1994) and Maltby, (1995). Therefore, *A. aquaticus* (studied in the field) may be inherently more resistant to pollution as opposed to *Gammarus* species studied in the laboratory.

Whilst noting the limitations of the comparison of different species, at similar endpoints and dose rates the present study found significant differences between the responses of organisms exposed to radiation in the lab (Chapter 2) as compared to those exposed in the natural environment (Chapters 3 & 4). Our crustacean data does not support the hypothesis that organisms in contaminated environments show greater radiation effects than those exposed in laboratory settings. These findings challenge the available data within the field of radioecology (e.g. Garnier-Laplace et al., 2013) and have important implications for assessment of radiation risk in the environment. The vast majority of data used to derive reference and benchmark levels for safe exposure are derived from single-species acute exposures conducted in the lab. Based on our data, these studies may provide overly-conservative estimates of risk posed by chronic radiation exposure in the natural environment, particularly considering that regulatory benchmarks are set to protect wildlife populations, whereas most laboratory studies consider effects on individuals. Our study emphasises the continued importance of conducting field monitoring in contaminated environments to determine environmental risk (Murphy et al., 2011). Future studies should focus on elucidating the drivers of the observed differences between laboratory and natural environment exposures using mesocosm studies.

6.2 Is Chronic Exposure to Low Levels of Radiation in the Environment Significantly Impacting Crustaceans in Contaminated Sites?

In Chapter 1, one of the aims of the present PhD project was 'To determine whether chronic exposure to low levels of radiation in the environment is significantly impacting crustaceans in contaminated sites'. To answer this question, two crustacean species were collected from highly contaminated areas at Chernobyl and Fukushima and effects on a range of endpoints including development, reproduction and genetic diversity were assessed. No significant effects were found for any of the previously mentioned endpoints in crustaceans at Chernobyl (developmental, reproductive and genetic diversity effects) or Fukushima (developmental effects only). Based on the evidence presented in this PhD thesis, it is therefore concluded that current dose rates at Chernobyl and Fukushima are not inducing significant effects on crustaceans. However, it is important to consider that significant effects may have occurred previously (particularly at the much higher dose rates shortly after the accident) at either the individual or population level but may have recovered over time.

This study has provided novel evidence regarding the effects of radiation on crustaceans exposed in the natural environment. Despite thirty years of research, the effects of chronic radiation exposure on biota remain unclear and are controversial within the scientific literature (see Beresford et al., 2016). This is in part owing to a significant number of studies reporting severe effects in biota at dose rates lower than is expected from the available radioecology evidence (e.g. Møller & Mousseau, 2009). Radioecology studies often underpin risk assessments, regulatory legislation and the efficacy of remediation efforts in contaminated areas. The novel finding of no significant effect of radiation exposure on crustaceans following long-term radiation exposure in the natural environment will aid in assessing the risk posed by radiation in the aquatic environment and the future management of radioactively contaminated areas.

6.3 Relevance for Environmental Radioprotection

One of the key aims of the present PhD project as outlined in Chapter 1 was 'To assess the efficacy of current proposed benchmarks for protection of the environment from ionising radiation'. The current proposed dose rates for protection of the environment by various organisations are shown in Table 1.2. In the United Kingdom, a screening dose rate of 40 µGy/hr is used by the Environment Agency (Copplestone et al., 2001), above which a more site-specific assessment is necessary. Proposed values wherein deleterious effects are anticipated to occur for aquatic organisms are significantly higher, generally being in the range of 10 – 100 mGy/d or approximately 400 to 4000 µGy/hr (e.g. IAEA, 1992; ICRP, 2008; UNSCEAR, 1996). These values are typically based on critical reviews of the available radiation effect literature or statistical techniques using reconstructed dose-effect relationships (Garnier-Laplace et al., 2006). There are a number of limitations associated with these methods, including the lack of environmentaly relevant exposure data for many organisms. Though recent studies have shown a trend toward the use of environmentally relevant dose rates in radioecology studies (Dallas et al., 2012), the majority of published studies are based on acute exposures to high doses of radiation.

In laboratory studies (see Chapter 2), significant effects on crustacean male fertiliy were recorded at doses of 1 mGy/d (~ 42 µGy/hr). Evidence for effects at lower doses of 0.1 mGy/d (~ 4.2 µGy/hr) were also observed, though this is subject to some uncertainty (see discussion in Chapter 2). Based on the laboratory data, our study suggests that current proposed benchmark values wherein effects are expected to occur (e.g. the ICRPs DCRL values) may not be adequate. From a regulatory perspective, effects at the population-level are considered the most significant for environmental protection. The effects observed in the laboratory component of the present research (e.g. a reduction in sperm quality with knock-on impacts on the number and development of progeny) would be anticipated to have population-level effects. Ford et al., (2012) used a dynamic population model to emphasise the potential of subtle perturbations to male fertility in amphipod crustaceans to have significant population-level consequences. Based on data for the brackish water amphipod, *Gammarus duebenii*, a 5 – 10% reduction in the number of sperm and the associated reduction in brood sizes would cause a crash in the population size within six years (Ford et al., 2012). However, extrapolating laboratory toxicity data measured at the individual level to higher population level effects in the field is subject to significant uncertainty (Kramer et al., 2011). Future research into the consequences of the effects observed in the present study for crustacean populations is necessary to draw direct conclusions about the appropriateness of the proposed benchmarks.

From a regulatory context, it is important to consider that dose rates at which effects were found to occur in the present study remain significantly higher than typical environmental exposures. Effects were recorded at dose rates of 1 mGy/d, equivalent to 41.7 µGy/hr. Calculated dose rates to *Asellus aquaticus* at Glubokoye lake (assessed in 2015), often considered the most radioactively contaminated water body in the world, were 27.1 µGy/hr. Doses received by biota from permitted releases at nuclear facilities are orders of magnitude lower. For example, in the cooling pond of the Ignalina NPP (Lithuania), doses received by crustaceans from anthropogenic radionuclides were found to be as low as 0.012 µGy/hr, significantly lower than background radiation doses within the same site (Nedveckaite et al., 2011). This suggests that though effects may be recorded below some of the proposed environmental protection benchmarks, it is unlikely that significant effects are occurring in the natural environment based on our data.

In conclusion, the present work suggests that effects may occur at lower doses for some organisms than predicted by regulatory organisations (generally in the range of 10 – 100 mGy/d or approximately 400 to 4000 µGy/hr e.g. IAEA, 1992; ICRP, 2008; UNSCEAR, 1996). However, this is unlikely to impact upon the system for environmental radioprotection due to the limited number of exposure scenarios wherein such dose rates are recorded. The present study does, however, emphasise the continued need for species-specific data regarding the effects of environmentally relevant doses of radiation on organisms as benchmarks derived largely from acute high dose studies may lead to underestimation of risk.

6.4 Relevance for Environmental Toxicology

A comparison of the findings of the present study with laboratory studies of environmentally relevant exposures to other pollutants in amphipod crustaceans is shown in Table 6.1. This was conducted to determine the relative importance of radionuclide effects on organisms in comparison with other contaminants widely abundant in the environment. Dose levels at which effects were found to occur in the present study were 1 mGy/d in the laboratory (see Chapter 2), with no evidence for effects at doses up to 0.92 mGy/d (38.1 µGy/hr) in the field (see Chapters 3, 4 and 5). For other pollutants, e.g. endocrine disruptors, low doses of 0.05 and 5 µg/l have been shown to have deleterious effects on *Gammarus* species in the lab (see Table 6.1). Such concentrations are environmentally relevant and are within the range of those encountered in the environment. For example, Green et al., (2015) modelled concentrations of pharmaceuticals in river catchments across the UK based on prescriptions and per capita excretion rates. The authors predicted concentrations of Cyproterone acetate occurring in the range of 0.01 – 0.05 µg/l in the majority of the fourteen UK river catchments studied. In *G. pulex,* Gismondi et al., (2017) demonstrated a significant reduction in the number of sperm in individuals exposed to 0.05 µg/l (see Table 6.1).

Comparatively, the present study focused on radiation effects on biota collected from sites considered amongst the most radioactively contaminated in the world and dose rates in the laboratory representative of those environments. For example, maximum dose rates in the field were 0.92 mGy/d which was calculated for crustaceans inhabiting sites less than 5km from the Fukushima nuclear plant, with no significant effects recorded. It is important to consider that these dose rates are orders of magnitude greater than typical environmental exposures from background radiation and permitted discharges from nuclear facilities (see above Section 6.3). Based on the frequency of scenarios wherein organisms are likely to encounter contaminant levels shown to cause negative effects, the severity of the risk posed by radiation in the environment may be limited in scale as compared to other environmental contaminants. However, this comparison is based on limited data and must be interpreted with caution owing to differences in the specific experiment design, specific endpoints use and mode of action of the given contaminant. Furthermore, elevated dose rates at areas such as Chernobyl and Fukushima will persist for many years to come. Coupled with the ongoing scientific debate regarding the long-term impacts of low dose radiation, this emphasises the continued need for monitoring of the impacts of radiation on wildlife.

Table 6.1 – Summary of studies in amphipod crustaceans assessing the impacts of environmentally relevant contaminant exposure. The results are shown compared to the findings of the present study.

Toxicant	Type of Contaminant	Test Species	Effect Studied	Lowest Observed Effect Level (LOEL)	Laboratory or Field	Reference
Cadmium	Heavy Metal	*Gammarus fossarum*	Number of Spermatozoa	1 µg/l	Laboratory	Trapp et al., (2015)
Cadmium	Heavy Metal	*Gammarus fossarum*	Embryo Abnormalities	3 µg/l	Laboratory	Geffard et al., (2010)
Copper	Heavy Metal	*Gammarus aequicauda*	Growth	50 µg/l	Laboratory	Prato et al., (2013)
Pyriproxyfen	Endocrine Disruptor	*Gammarus fossarum*	Number of Spermatozoa	5 µg/l	Laboratory	Trapp et al., (2015)
Cyproterone Acetate	Endocrine Disruptor	*Gammarus pulex*	Number of Spermatozoa	0.05 µg/l	Laboratory	Gismondi et al., (2017)
Simvastin	Pharmaceutical Drug	*Gammarus locusta*	Number of newborns per female	0.32 µg/l	Laboratory	Neuparth et al., (2014)
Phosphorus-32	Radionuclide	*Echinogammarus marinus*	Sperm Viability	1 mGy/d	Laboratory	This thesis
Phosphorus-32	Radionuclide	*Gammarus pulex*	Sperm Viability	No Significant Effect	Laboratory	This thesis

6.5 Future Perspectives & Conclusions of the Project

The present research has adopted a combined laboratory and field approach to assessing the effects of radiation on crustaceans. In Chapter 1, a comprehensive review of the current understanding of radiation effects on crustaceans was provided. Key findings were the paucity of data across all endpoints and particularly in respect to radiation effects on crustaceans in the natural environment. In a controlled laboratory exposure, Chapter 2 focused on the effects of environmentally relevant doses of radiation on male fertility, sperm DNA damage and reproduction in marine and freshwater crustaceans. Sperm viability was demonstrated to be a useful endpoint and was significantly reduced following radiation exposure in the marine crustacean. In the same species, subsequent experiments recorded an increase in sperm DNA damage which was found to be related to elevated incidence of embryo abnormalities and reduced fecundity following reproduction with an unexposed female.

Whilst this research has substantially increased our understanding of the effects of radiation on crustaceans, it has raised a number of questions that future research should aim to address. For example, the goal of environmental protection is often stated to be maintenance of populations of organisms. Though significant individual level effects were recorded at dose levels below expected, the implications for populations of individuals remain unclear. Future research should therefore focus on linking effects at the individual level with studies of populations to determine the relevance of the results of the present study at higher levels of ecological organisation.

Structured population models have been proposed as one method of translating individual radiation effects to higher levels. Alonzo et al., (2008b) modelled the effects of chronic radiation exposure on *Daphnia magna* and the earthworm *Eisenia fetida* using simple population models and data obtained from chronic exposure experiments. The authors emphasised the importance of ranking the sensitivity of population growth to a number of different endpoints to adequately assess risk using this method. Furthermore, single generation studies were found to underestimate risk, emphasising the need for longer term multigenerational studies. Though population models for the study species in the present research exist (see Ford et al., 2012; Martins et al., 2002), the efficacy of modelling for accurate risk assessment would be enhanced by multigenerational radiation exposures and a broader range of endpoints studied. This would allow for greater predictive power in determining the relevance of the results of the present study to natural crustacean populations and aid in environmental radioprotection.

Chapter 3 focused on the effects of chronic radiation exposure at Chernobyl and Fukushima on the developmental stability of two crustacean species, *Asellus aquaticus* and *Eriocheir japonica* measured using fluctuating asymmetry. No significant effect of chronic radiation exposure on developmental stability of either species was recorded. This study is the first to consider effects of chronic radiation exposure on crustaceans, internationally important model organisms in radioecology, at Chernobyl and Fukushima. To the author’s knowledge, this is also the first study of radiation effects in any aquatic biota at Fukushima. This data will be implemented by international radiological protection groups (e.g. ICRP, IAEA) in future environmental risk assessments and aid in the management of contaminated environments. Though the present study comprehensively analysed the effects of chronic radiation exposure on fluctuating asymmetry, future research should continue to monitor a range of other endpoints reflecting individual fitness. Fluctuating asymmetry has been shown in numerous studies to be a useful indicator of radiation exposure (see Chapter 3 for further discussion); however its direct relationship with fitness has been criticised (Lens et al., 2002). Therefore, future studies should employ more direct measurements of fitness in conducting continued monitoring of biota in radioactively contaminated environments.

Reproduction in the freshwater crustacean, *Asellus aquaticus*, along a gradient of contamination at Chernobyl was studied in Chapter 4. Over two sampling years, no significant effect of chronic radiation exposure on fecundity was recorded. This finding has important implications for the long term maintenance of aquatic invertebrate populations in radioactively contaminated environments and adds to the body of evidence presented in Chapter 3 regarding effects on development. The finding of no significant effect on these endpoints does not indicate that no negative effects are occurring on crustaceans in radioactively contaminated environments. As highlighted in Chapter 4, resources may be diverted away from other processes to maintain developmental stability and normal reproductive output despite chronic radiation exposure. Changes to available energy resources are often recorded in individuals inhabiting chronically polluted environments (e.g. Durou et al., 2007; Smolders et al., 2004; Vijayavel et al., 2006). Furthermore, controlled laboratory exposures of *Daphnia magna* to radiation have recorded alterations to metabolic expenditure resulting in reduced scope-for-growth (Alonzo et al., 2006), suggesting energy budgets may be impacted by radiation. Future research should adopt a biochemical approach to measure energy reserves in biota inhabiting radioactively contaminated environments.

In Chapter 5, the impact of chronic radiation exposure on genetic diversity of *Asellus aquaticus* at Chernobyl was studied. No significant effect of radiation was recorded, contradicting the majority of studies suggesting elevated genetic diversity in biota at Chernobyl. This study is the first to consider the impacts of radiation on genetic diversity in an aquatic invertebrate and the first to use next-generation techniques to assess genetic diversity at Chernobyl. Given the host of advantages associated with these next generation techniques compared to previous methods (see Chapter 5 for further discussion), this study represents a significant advancement in understanding the long-term genetic implications of radiation exposure. However, continued demographic study of aquatic populations is necessary to support and contextualise these findings. An understanding of key demographic parameters such as survival, recruitment and population density are fundamental to interpreting and contextualising population genetic data (Lowe et al., 2017). Future research should monitor demographic processes in aquatic invertebrates in Chernobyl lakes to enable future predictions regarding the success of aquatic populations in contaminated environments.

In conclusion, the present research has adopted a combined laboratory and field approach to provide a robust assessment of the effects of ionising radiation on a previously understudied group of organisms. Given the international importance of crustaceans as models in the field of environmental radioactivity (ICRP, 2008), the novel data presented throughout this thesis will substantially contribute toward the unified system for protection of the environment from radiation. This study has challenged the current understanding in radioecology regarding the differences between laboratory and field studies. In the laboratory, evidence was provided for effects at dose rates below expected based on the available radioecology data, though significantly higher than typical environmental exposures. Conversely, field studies of crustaceans in contaminated environments at Chernobyl and Fukushima found no significant effects of radiation on a range of endpoints including development, reproduction and genetic diversity. Given the importance of controlled laboratory exposures in determining environmental protection benchmarks, the present study has raised fundamental questions regarding the efficacy of laboratory studies for environmental protection that will shape future research.

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Appendix

Appendix A – *Antisymmetry Tests*

Table A1. – Kurtosis statistics of each lake x trait combination in *Asellus aquaticus*

	Gorova	Rita River	Orekhovskiy Channel	Dvorische	Stoyecheye	Svatoye 3 (2015)	Svatoye 3 (2004)	Perstok	Yanovsky Crawl	Glubokoye (2015)	Glubokoye (2004)
First Antennae	-0.113	.261	.945	1.348	0.963	2.743	.762	.310	3.794	2.456	4.253
Propodos Width	1.974	.167	2.506	0.702	0.278	1.851	-.108	.903	1.173	3.151	2.070
Merus Width	2.923	.542	1.384	0.035	-0.242	-0.642	.726	2.333	0.824	0.879	.485
Carpus Width	0.128	.727	.180	0.507	0.087	0.641	.806	-.137	0.807	0.763	.476

Table A2. – Kurtosis statistics of each lake x trait combination in *Eriocheir japonica*. Numbers in bold represent significant non-normal traits (Kolmogorov-Smirnov test, $p < 0.05$)

	Matsukawa-ura	Niida River	Ukedo River	Funazawa
Fourth Pereiopod Merus Length	-0.494	.788	2.393	4.166
Fourth Pereiopod Merus Width	3.572	2.006	4.433	3.539
Third Pereiopod Carpus Length	1.651	-.492	-0.467	-0.506
Third Pereiopod Carpus Width	2.028	2.850	0.470	1.665
Second Periopod Propodos Length	3.400	2.979	2.116	-0.176
Second Pereiopod Propodos Width	**14.641**	2.641	**7.866**	3.911
First Pereiopod Merus Length	**4.787**	1.275	.817	0.432
First Pereiopod Merus Width	**8.374**	3.638	**12. 509**	0.062

Appendix B - *Publications*

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The biological effects of ionising radiation on Crustaceans: A review

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ABSTRACT

Historic approaches to radiation protection are founded on the conjecture that measures to safeguard humans are adequate to protect non-human organisms. This view is disparate with other toxicants wherein well-developed frameworks exist to minimise exposure of biota. Significant data gaps for many organisms, coupled with high profile nuclear incidents such as Chernobyl and Fukushima, have prompted the re-evaluation of our approach toward environmental radioprotection. Elucidating the impacts of radiation on biota has been identified as priority area for future research within both scientific and regulatory communities. The crustaceans are ubiquitous in aquatic ecosystems, comprising greater than 66,000 species of ecological and commercial importance. This paper aims to assess the available literature of radiation-induced effects within this subphylum and identify knowledge gaps. A literature search was conducted pertaining to radiation effects on four endpoints as stipulated by a number of regulatory bodies: mortality, morbidity, reproduction and mutation. A major finding of this review was the paucity of data regarding the effects of environmentally relevant radiation doses on crustacean biology. Extremely few studies utilising chronic exposure durations or wild populations were found across all four endpoints. The dose levels at which effects occur was found to vary by orders of magnitude thus presenting difficulties in developing phyla-specific benchmark values and reference levels for radioprotection. Based on the limited data, mutation was found to be the most sensitive endpoint of radiation exposure, with mortality the least sensitive. Current phyla-specific dose levels and limits proposed by major regulatory bodies were found to be inadequate to protect species across a range of endpoints including morbidity, mutation and reproduction and examples are discussed within. These findings serve to prioritise areas for future research that will significantly advance understanding of radiation-induced effects in aquatic invertebrates and consequently enhance ability to predict the impacts of radioactive releases on the environment.

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1. Introduction

The renewed interest in nuclear power as a low carbon emission energy source coupled with concern regarding past and potential nuclear accidents dictate that elucidating the impact of radionuclides on the environment is a global issue. Traditional approaches to radiological protection of the environment are based on the assumptions that the standards of environmental control needed to protect humans would be adequate to protect other species (Copplestone et al., 2004; International Commission on Radiological Protection (ICRP, 1977). However this anthropocentric approach is no longer accepted due to the paucity of information regarding the effects of ionising radiation on non-human biota (Pentreath, 1998; Thompson, 1988), the varying sensitivities of different species and developmental stages to radioactive contaminants (Hagger et al., 2005) and the existence of habitats in which organisms may be exposed to doses above the permissible limits for humans (Copplestone et al., 2001). Assessing the biological impact of ionising radiation on non-human biota has been identified as a necessary approach towards protecting and mitigating the impacts of future radioactive releases on the environment by a number of international directives (e.g., ERICA and PROTECT [Howard et al., 2010; Larsson, 2008]).

The presence of ionising radiation in the environment originates from both natural and anthropogenic sources. Natural sources include cosmic radiation originating from outside the solar system and primordial radionuclides arising from stellar processes (Smith & Beresford, 2005). The majority of anthropogenic radionuclides in the environment are derived from three major sources: nuclear weapons testing, nuclear disasters and permitted discharges from nuclear reprocessing plants (Aarkrog, 2003). The aquatic environment represents an important sink for radionuclides (Avery, 1996), since the majority of deposition of radioactive waste from nuclear facilities is in liquid form and deposition of atmospheric fallout in ocean ecosystems is approximately two-fold higher than in terrestrial systems (Burton, 1975). Radionuclides present in the terrestrial environment may also contribute to radioactivity in aquatic environments via run-off. For ^{137}Cs and ^{90}Sr, the two major man-made contributors to worldwide radiation doses (IAEA, 1995), approximately 2 and 9% of the total land inventories will be transported to aquatic systems respectively via this pathway (Yamagata et al., 1963). However, the bioavailability of radionuclides derived from run-off is often limited by the binding of such radionuclides to particulates and subsequent sedimentation (Aarkorg, 2003). Furthermore, catchment and soil characteristics have been demonstrated to significantly impact the mobility of radionuclides by this pathway (Smith et al., 2004).

Direct disposal of solid radioactive waste into the marine environment was conducted over a 48 year period from 1948 to 1993, leading to dumping of approximately 85 PBq (1×10^{15} Bq) of radioactive material (IAEA, 1999). The majority of dumped waste was low level solid waste deposited in the NE Atlantic and disposal of reactors by the former Soviet Union in the Kara Sea, being 53.4 and 43.3% of total dumped activity respectively (IAEA, 1999). Radiological monitoring of dump sites by a number of organisations revealed negligible impacts on overall radioactive contamination and emphasised the greater influence of atmospheric fallout, although elevated radionuclide levels were observed in the vicinity of some dump sites (i.e., Baxter et al., 1995).

Permitted releases from nuclear reprocessing sites represent a significant source of anthropogenic radionuclides to the world's oceans. For example, the Sellafield nuclear spent fuel reprocessing site located in Cumbria, United Kingdom, generated a liquid radioactive effluent of 6.649×10^5 GBq beta and gamma emitters (excluding tritium) over a four year period from 1995 to 1999 (European Commission, 2001). Such discharges are detectable in most areas of the NE Atlantic and in the Arctic Ocean, representing a significant transfer of radioactive contamination (Kershaw & Baxter, 1995). Major catastrophes such as the explosion at the Chernobyl NPP and the Tōhoku earthquake–tsunami at the Fukushima Dai-ichi NPP led to large scale releases of radioactive material into the environment (Buesseler et al., 2012). Estimates of the overall input of ^{137}Cs to the world's oceans as a consequence of the Chernobyl incident are 15–20 PBq (Aarkrog et al., 2003). Finally the use of radioisotopes in medical, industrial and scientific institutions leads to contamination of the marine environment typically orders of magnitude lower than other major sources (Aarkrog et al., 2003).

Although the need for environmental radioprotection frameworks has long been established, (Pentreath and Woodhead, 1988; Pentreath, 1998) a lack of scientific consensus regarding the doses at which significant biological effects occur (Beresford & Copplestone, 2011) and the disparity between results of laboratory based exposures and field studies (Garnier-Laplace et al., 2013) have precluded a radiological risk assessment for the environment. This provides a contrast with other anthropogenic contaminants wherein protection frameworks and concepts (i.e., the Ecological Risk Assessment concept) are well developed (Bréchignac, 2003). An overview of the effects of ionising radiation on aquatic invertebrates has previously been carried out by Dallas et al., (2012). This paper adopts a phyla-specific approach in order to provide a more detailed analysis of effects, and prioritise research needs for the Crustacea, a group of organisms that have been identified as key models for the development of environmental radioprotection frameworks (ICRP, 2008). Members of the subphylum Crustacea are the dominant components of global aquatic ecosystems and comprise more than 66,000 species (LeBlanc, 2007). These organisms provide an array of commercial and ecological services and are used both directly for human consumption and as a food source for other commercially important species (Benzie, 2009). Due to their ubiquity in aquatic environments and well characterized biology, a marine crustacean of the family Cancridae has been selected as one of the ICRP's reference animals (ICRP, 2008). Reference organisms will be used as a basis to develop environmental radioprotection measures, and are considered ecologically representative of a specified group of plants or animals with biological characteristics amenable to study (ICRP, 2008). To support the development of robust, applicable ecological benchmark values for environmental radioprotection it is necessary to review and identify research needs for radiobiological studies in the selected reference organisms. This paper aims to review the available literature regarding the biological effects of ionising radiation on the crustacean subphylum, draw comparisons across biomarkers and assess any gaps in knowledge in the context of developing dose levels for radioprotection. Emphasis will be placed on studies employing the four biological endpoints as outlined by Copplestone et al., (2008) and Real et al., (2004); mutation, morbidity, reproductive capacity and mortality.

The scope of the literature search was limited to aquatic crustacean species exposed to any form of ionising radiation. The FREDERICA radiation effects database (Available at http://www.frederica-online.org) along with other search engines (Google Scholar, Science Direct and Web of Science) was used as a tool to extract references relating to the four umbrella endpoints selected in this review. The FREDERICA database contains references from a number of European Commission funded projects (i.e., EPIC and FASSET) from 1945 to 2007. All references within this dataset are subject to review based on the adequacy and reproducibility of the study (Copplestone et al., 2008).

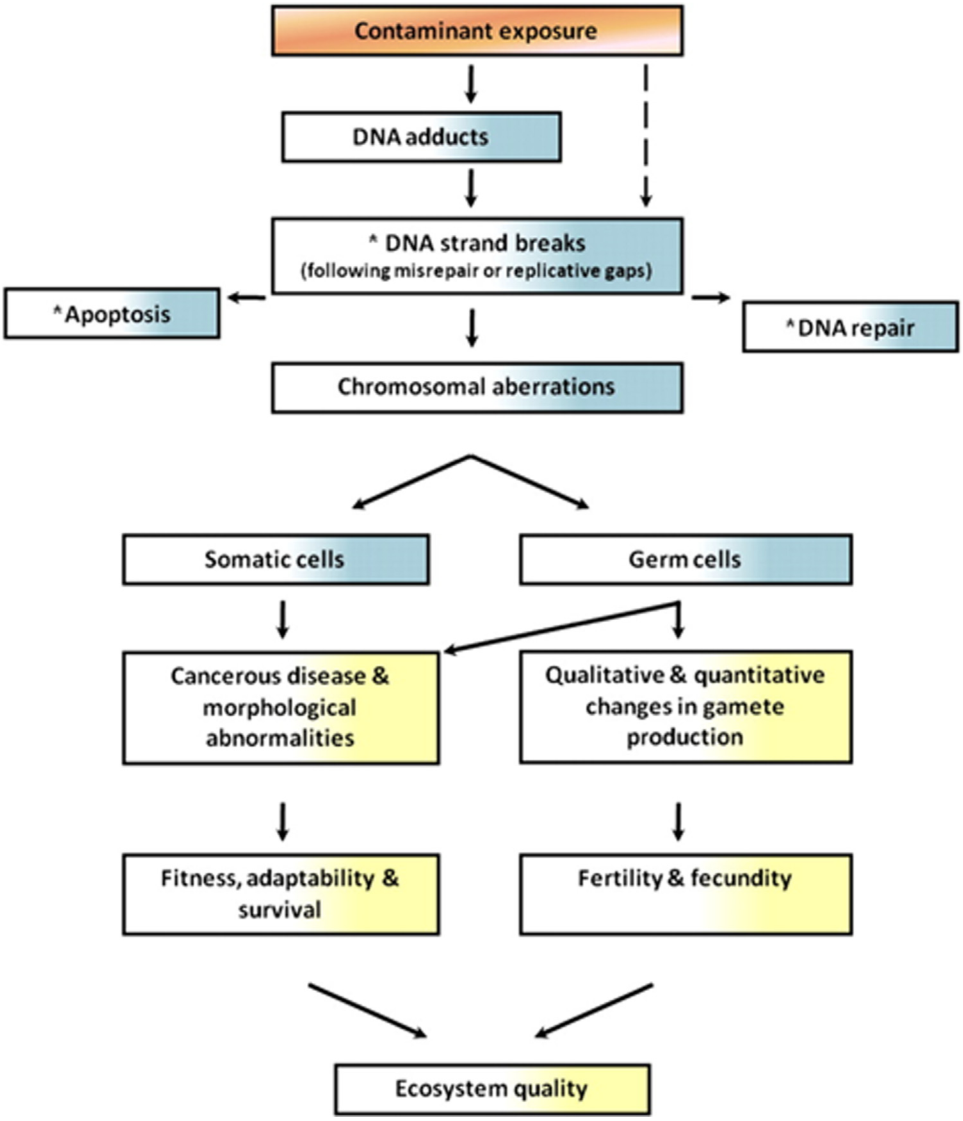

Fig. 1. Illustration of the relationship between contaminant exposure and ecosystem quality. Adapted from Jha (2008). Fig. 1 reproduced with permission from Jha (2008).

2. Radiation-induced mutation in crustaceans

For the purpose of this review, a mutation is defined as "A change in the chromosome or genes of a cell which may affect the structure and development of the resultant offspring" (Copplestone, 2008). Chromosomal and genetic changes have been postulated by a number of authors to have significant ecological implications at higher levels of biological organisation (See Fig. 1 (Anderson and Wild, 1994; Depledge, 1998; Jha, 2008).

Despite evidence suggesting the clastogenic (capacity to cause chromosomal aberrations) and mutagenic potential of ionising radiation observed in a range of organisms including humans (Lucas et al., 1992), fish (Anbumani and Mohankumar, 2012; Kligerman et al., 1975; Pechkurenkov, 1991) and molluscs (AlAmri et al., 2012), there is a paucity of information within the literature regarding the crustacean subphylum. Indeed, the FREDERICA database containing over 30,000 data entries collated from a number of international radiation effects directives contains no data regarding mutation in crustacean species over chronic dose ranges of 0–>10,000 μGy/hr-1 (See Table 1 Copplestone et al., 2008). Similarly, the 2008 ICRP publication introducing the concept of reference animals and plants reported no available data for chromosomal effects in crab species (ICRP, 2008), reiterating the lack of studies in this area.

Field studies have suggested that mutation may be a sensitive endpoint of radiation-induced effects in crustacean species. For example, Florou et al., (2004) assessed chromosomal aberrations in microfauna collected from geothermal spring areas on the island of Ikaria, Greece where maximum dose rates of natural gamma emitters in sediments were 9.6 mGy yr^{-1} (~0.001 mGy/hr^{-1}). These values are substantially elevated above the reported mean of 0.07 mGy yr^{-1} (~0.008 μGy/hr^{-1}) for coastal sediments in Greece (Florou and Kritidis, 1992). An elevated level of cells displaying chromosome aberrations (3.8%) was recorded in populations of the amphipod crustacean *Melita palmata* collected from these areas compared with control sites (1.5–1.7%). The author (Florou et al., 2004) attributed this to increased natural dose rates of gamma and natural alpha emitters, which were also increased above background levels in spring areas (14–26 Bq l^{-1} of ^{222}Rn compared with 1.3–7 Bq l^{-1} in control areas). These dose values fall significantly below proposed environmental protection benchmark values provided by a number of organisations (See Table 2) suggesting induction of significant biological effects below doses that are considered to have no deleterious effects at the population level. However, the biota inhabiting geothermal spring habitats are typically species-poor and subject to multiple stressors including elevated temperatures in winter periods (Flourou et al., 2004)

Table 1
Collation of available chronic radiation effect data and data gaps within the subphylum Crustacea located in the FREDERICA Radiation Effects Database. X = available data - = no data available. Reproduced with permission of Copplestone et al., (2008).

	Dose Rate Range (μGy/hr^{-1})								
	0–50	50–100	100–200	200–400	400–600	600–1000	1000–5000	5000–10,000	>10,000
Morbidity	X	X	–	–	–	X	X	X	–
Mortality	–	–	–	–	–	–	–	–	–
Mutation	–	–	–	–	–	–	–	–	–
Reproductive Capacity	–	–	–	–	–	–	–	–	–

and extremes of pH and chemical toxicants (Duggan et al., 2007). Thus, the observed cytogenetic response in spring biota may have been due to the complex environmental conditions present at the study sites as opposed to the direct effects of ionising radiation. This highlights the inherent difficulties in field radioecology studies (Salbu, 2009) and the importance of quantifying the individual contribution of stressors in environments where abiotic pressures may act synergistically (Dallas et al., 2012). The aforementioned paper represents the only study of natural crustacean populations using mutation as an endpoint. The previously discussed limitations present difficulties in drawing conclusions from this study as observed cytogenetic effects cannot be directly attributed to ionising radiation.

Laboratory studies assessing radiation-induced mutations in crustaceans typically involve acute high doses that are unrepresentative of environmental exposures. Such studies have demonstrated the ability of ionising radiation to induce chromosomal aberrations in crustacean species. Tsytsugina (1998) exposed embryos of two crustacean species, *Idotea baltica* and *Gammarus olivii* to doses of 0.5–5 Gy from a range of radionuclides and chemical mutagens (Lead Acetate and Chlorophene) and scored cells on the presence of chromosomal abnormalities. The mean number of cells with chromosomal aberrations increased concomitant with radiation dose. Furthermore, the author described characteristic types of aberrations produced by the two toxicants which may be used to distinguish between the effects of individual stressors. For example, ionising radiation was shown to elicit chromosomal damage in the form of single and twin fragments, whereas, single and twin bridges were more commonly observed in those embryos exposed to chemical toxicants. The distribution of aberrations between cells was also found to correspond to different statistical distributions dependent on the toxicant, underpinning the potential of this method. However, the karyotype of crustacean species is often reported to be unamenable to cytogenetic study (Salemaa, 1985) due to the typically small size and high diploid numbers of chromosomes (White, 1973). This may preclude application of this method to natural populations.

Recent approaches to assessing radiation-induced genotoxicity in aquatic invertebrates have involved monitoring levels of the expression of genes that are involved in DNA damage repair pathways (AlAmri et al., 2012; Han et al., 2014a; Han et al., 2014b; Won and Lee, 2014). For example, Han et al., (2014b) exposed cultures of the intertidal copepod, *Tigriopus japonicus* to gamma radiation from ^{137}Cs and monitored mRNA expression of three DNA repair genes: *Ku70* (*Xrcc6*), *Ku80* (*Xrcc5*) and *DNA-PK*. These three genes are integral to the non-homologous end joining (NHEJ) DNA repair pathway involved in the detection and repair of radiation-induced double strand breaks (DSBs) (Mahaney et al., 2009). Expression of the three genes was significantly elevated with respect to controls in 200 Gy exposed organisms, suggesting induction of DSBs at these dose levels (Han et al., 2014b). The potential of this approach as a biomarker for genotoxicity in crustacean species was emphasised by Won and Lee (2014) who reported a dose dependent increase in mRNA expression of these genes in another copepod species, *Paracyclopina nana* (See Fig. 2). However, both of these studies used dose levels significantly higher than those encountered in radioactively contaminated environments (except perhaps in the immediate aftermath of a major nuclear accident). Furthermore, in the former study (Han et al., 2014b) gene expression was only monitored at dose levels of 150 and 200 Gy, despite induction of significant biological impacts such as a reduced fecundity in *T. japonicus* at three-fold lower dose levels (50 Gy). Alterations to gene expression patterns and molecular level responses are often reported to be sensitive indicators of contaminant exposure in aquatic invertebrate species (Lee et al., 2006). A recent study supported the previous statement, reporting significant DNA alterations in *Daphnia magna* following exposure to ^{137}Cs doses as low as 0.007 mGy h^{-1} using random amplified polymorphic DNA-polymerase chain reaction (RAPD–PCR) methods (Parisot et al., 2015). Molecular level responses were evident at both lower doses and shorter exposure durations than other endpoints including mortality, morbidity and perturbations to reproduction. Given the sensitivity of molecular endpoints to ionising radiation observed in this publication, it is imperative that a greater number of studies focus on molecular alterations in relation to effects at higher levels of biological organisation to confirm these findings within the crustacean subphylum.

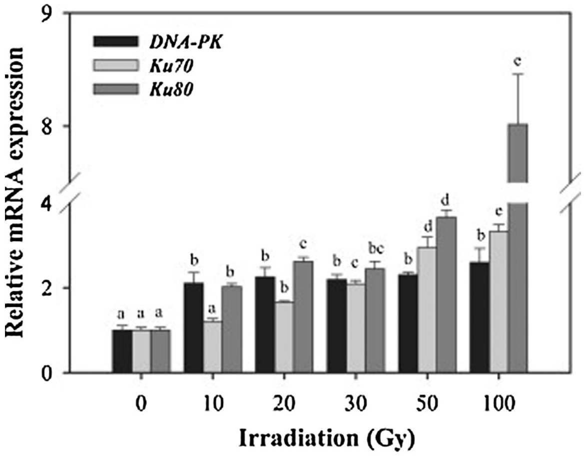

Fig. 2. Dose-dependent increase in mRNA expression of three genes involved in the non-homologous end joining pathway for DNA repair in gamma radiation exposed cultures of the copepod, *Paracyclopina nana*. Fig. 2 reproduced with permission of Won & Lee (2014).

3. Radiation impacts on morbidity in Crustaceans

Morbidity can be broadly defined as "A loss of functional capacities generally manifested as reduced fitness, which may render organisms less competitive and more susceptible to other stressors, thus reducing their life span" (Copplestone et al., 2008). Definition of the term morbidity varies between authors and encompasses a vast number of endpoints including perturbations to growth rates, behavioural alterations and immune system effects (Copplestone et al., 2004). In order to maintain relevance both ecologically and for environmental protection, an endpoint should be amenable to

Table 2

Numerical benchmark values in μGy/hr^{-1} proposed by a number of different organisations and directives for the protection of populations of a range of biota. US DOE = United States Department of Energy. NCRP = National Council on Radiation Protection and Measurements. IAEA = International Atomic Energy Agency. - = No data provided. Adapted from Andersson et al., (2008).

Dose Level (μGy/h^{-1})									
	US DOE (1990)	NCRP (1990)	IAEA (1992)	Environment Canada (2003)	FASSET (2003) Larsson, (2004)	ERICA (2007) Beresford et al. (2007)	ICRP (2008)	UNSCEAR (2008)	PROTECT (2009) Andersson, (2008)
Freshwater Organisms	400	400	400	–	100	10	–	400	10
Algae	–	–	–	100	–	–	–	–	–
Macrophytes	–	–	–	100	–	–	–	–	–
Benthic Invertebrates	–	–	–	200	–	–	–	–	–
Fish	–	–	–	20	–	–	–	–	–
Reference Trout	–	–	–	–	–	–	40–400	–	–
Reference Frog	–	–	–	–	–	–	4–40	–	–
Marine Organisms	400	400	–	–	100	10	–	400	–
Marine Mammals	–	–	–	–	–	–	–	–	–
Deep Ocean Organisms	–	–	1000	–	–	10	–	–	–
Reference Crab	–	–	–	–	–	–	400–4000	–	–
Reference Flatfish	–	–	–	–	–	–	40–400	–	–
Reference Brown Seaweed	–	–	–	–	–	40–400	–	–	
Terrestrial Organisms	–	–	–	100	100	10	–	100	10
Plants	–	–	400	–	–	–	–	–	–
Reference Pine Tree	–	–	–	–	–	–	4–40	–	–
Reference Wild Grass	–	–	–	–	–	–	40–400	–	–
Animals	–	–	40	–	–	–	–	–	–
Invertebrates	–	–	–	200	–	–	–	–	–
Reference Bee	–	–	–	–	–	–	400–4000	–	–
Reference Earthworm	–	–	–	–	–	–	400–4000	–	–
Mammals	–	–	–	100	–	–	–	–	–
Reference Deer	–	–	–	–	–	–	4–40	–	–
Reference Rat	–	–	–	–	–	–	4–40	–	–
Birds	–	–	–	–	–	–	4–40	–	–
Reference Duck	–	–	–	–	–	–	4–40	–	–

Table 3
Summary of morbidity studies in Crustacea. HTO represents Tritiated Water. Acute exposures are defined here as those lasting less than 24 h, with chronic exposures lasting over a period of the organisms life span and greater than 24 h

Species	Dose Rate/Total Dose	Lowest Observed Effect Dose/ Dose Rate (LOEDR)	Radiation Source	Exposure Duration	Exposure Type	Conclusion	Reference
Pollicipes polymerus	7.9, 62.5 nGy/hr^{-1}, 0.625, 6.25 and 62.5 μGy/hr^{-1}	0.000625 mGy/h^{-1}	HTO	32 Days	Chronic	Altered moulting patterns	Abbott & Mix, (1979)
Daphnia magna	0.02, 0.11 and 0.99 mGy/hr^{-1}	0.11 mGy/h^{-1}	^{241}Am	23 Days	Chronic	Reduction in body mass, Increased respiratory demand and Reduction in offspring fitness	Alonzo et al., (2006)
Daphnia magna	0.3, 1.5 and 15 mGy/hr^{-1}	0.3 mGy/h^{-1}	^{241}Am	70 Days	Chronic	Increased oxygen consumption, Reduction in body size and mass across generations	Alonzo et al., (2008a)
Artemia salina	100, 200, 400 and 800 Gy	200 Gy	$^{6\circ}$Co	~30–220 Minutes	Acute	Decrease in respiration rate	Angelovic & Engel (1968)
Callinectes sapidus	40, 80, 160, 320 and 640 Gy	40 Gy	$^{6\circ}$Co	~11–175 Minutes	Acute	Behavioural changes; reduction in irritability, catatonic state at high doses	Engel, (1967)
Daphnia magna	0.41, 4.2 and 31 mGy/hr-1	31 mGy/hr^{-1}	^{137}Cs	23 Days	Chronic	Decrease in mass-specific respiration rate, Reduction in offspring fitness	Gilbin et al., (2008)
Nephrops norvegicus	0.5 and 5 Gy	0.5 Gy	$^{6\circ}$Co	<5 Minutes	Acute	Morphological aberrations; deterioration of cytoplasm and aberrations in cytoplasmic organelles	Mothersill et al., (2001)
Daphnia magna	0.007, 0.07, 0.65, 4.7 and 35.4 mGy/hr^{1}	4.7 mGy/hr^{-1}	^{137}Cs	75 Days	Chronic	Reductions in body length and Von Bertalanffy growth rate	Parisot et al., (2015)
Palaemonetes pugio & *Uca pugnax*	9.75, 19.5, 48.75, 97.5, 195 and 390 Gy	9.1 Gy	$^{6\circ}$Co	0–20 Minutes	Acute	Alterations to moulting patterns	Rees, (1962)
Pacifastacus leniusculus trowbridgii	2.8, 5.6, 8.4,11.2 and 16.8 Gy	5.6 Gy	X-Ray	1 Minute	Acute	Behavioural changes; detection and avoidance of radiation source	Rodriguez & Kimeldorf (1976)
Macrobrachium rosenbergii	3,30,300 and 3000 mGy	3 mGy	$^{6\circ}$Co	~0–10 Minutes	Acute	Behavioural changes; alterations to swimming patterns; histological aberrations to the gill	Stalin et al. (2013a)
Macrobrachium rosenbergii	3,30,300 and 3000 mGy	3 mGy	$^{6\circ}$Co	~0–10 Minutes	Acute	Morphological deformations, Decreased hepatosomatic index	Stalin et al. (2013b)

measurement, specific to the hazard in question and appropriate for extrapolation to higher levels of biological organisation (Ankley et al., 2010; Suter, 1990). The practicality of using morbidity as an endpoint for radiation exposure may therefore be limited due to the lack of specificity and multitude of effects it includes. This is exemplified within the crustacean subphylum, with a diverse array of endpoints (See Table 3 for summary) used to assess morbidity.

3.1. *Radiation-induced impacts on growth & respiration*

Alonzo et al., (2006, 2008a) investigated the effects of chronic internal exposure to the alpha emitting radionuclide, ^{241}Americium, on the growth dynamics of *Daphnia magna*. The authors recorded a significantly lower dry mass and body length of irradiated specimens at doses of ~1.5 mGy/hr^{-1} in first generation organisms (F0), with significant increases in the severity of effects over generations. For example, individuals of the F2 generation displayed a 15% reduction in dry mass at doses of 0.3 mGy/hr^{-1} (Alonzo et al., 2008a). A recent study further underpinned the potential of ionising radiations to perturb growth dynamics in daphnids (Parisot et al., 2015), with reductions of 5 and 13 % in the growth rate of F2 generation daphnids exposed to 4.7 and 35.4 mGy/hr^{-1} of gamma radiation, respectively. Experimental evidence suggests that larger daphnids have enhanced competitive and resource exploitation ability relative to smaller individuals, leading to elevated mortality in those individuals with reduced competition capacity (Kreutzer and Lampert, 1999). The finding that radionuclide exposure may perturb growth dynamics therefore has important implications for natural crustacean population dynamics.

In the previous study (Alonzo et al., 2008a), oxygen consumption of *D. magna* was elevated above controls at all doses, suggesting an increase in metabolic expenditure induced by radiation stress. Exposure of organisms to stressors and adverse conditions may result in reallocation of metabolic energy towards maintenance and lead to reduced energy investment per offspring (Baillieul et al., 2005). This was reflected by a reduced resistance to starvation recorded in neonates derived from 0.02 mGy/hr^{-1} exposed adult daphnids (Alonzo et al., 2006). It is of note that this dose rate falls below the value of ~0.4 mGy/hr^{-1} provided by a number of organisations below which no deleterious population level effects are predicted to occur in aquatic organisms (See Table 2) by an order of magnitude. A recent study (Sarapultseva and Gorski, 2013) further suggested deleterious impacts on neonates relating to metabolic perturbations. Following parental exposure to acute gamma doses of 100 and 1000 mGy from Cobalt-60, a ~20% decrease in the mean life span of non-exposed first generation *D. magna* offspring was demonstrated.

Another study of *Daphnia magna* exposed to chronic gamma irradiation from ^{137}Cs reported contrasting results to the aforementioned study (Alonzo et al., 2008a) of decreased oxygen consumption with increasing dose (Gilbin et al., 2008). *D. magna* receiving gamma dose rates of 31 mGy/hr^{-1} displayed a significantly lower mass-specific respiration rate, compared with dose rates of 0.3, 1.5 and 15 mGy/hr^{-1} all eliciting an increase in respiration rate following Americium-241 (an alpha emitter) exposure in the study of Alonzo et al., (2008a). Whilst the low number of replicates ($n = 6$) recognised by the author in the study of Gilbin et al., (2008) may prevent comparison across studies, this underpins the importance of accounting for differing radiation sources and the corresponding variability in relative biological effectiveness (RBE). The term RBE was coined in 1931 (Failla and Henshaw, 1931) to account for the variability in biological effect observed with dose, dose rate and type of radiation (Valentin, 2003). RBE increases as a function of LET with high linear energy transfer (LET) sources of radiation, e.g., alpha emitters, typically more effective at eliciting biological damage in experimental systems than low LET radiation, i.e., gamma and beta rays reaching a maximum at ~100 keV/μm (Hall and Hei, 2003; UNSCEAR, 1996). This may be used to account for the different responses of *D. magna* in these two studies.

The variability in biological effect relating to the given radiation source is exemplified by a study of morbidity in the goose barnacle, *Pollicipes polymerus*, which recorded altered moulting patterns at extremely low beta doses of 0.62 μGy/hr^{-1} (Abbott and Mix, 1979). The radiation source employed in the previous study was tritiated water (HTO), a radionuclide that is discharged into groundwater systems from nuclear operations (Jaeschke et al., 2011; Jha et al., 2005). Despite the relatively low energy emission of beta particles from HTO (average beta energy of 5.73 ± 0.03 keV (Pillinger et al., 1961), the nature and behaviour of this radiation source within organisms has led to significant concern over the RBE of the radionuclide (Bridges, 2008; Little and Lambert, 2008). It has been demonstrated that HTO may be irreversibly incorporated into organic compounds within organisms (Takeda and Kasida, 1979) and therefore may produce a biological effect disparate with its emission characteristics. In addition, the authors of the aforementioned study on *Pollicipes polymerus* (Abbot and Mix, 1979) stated that calculated doses were exclusive of background radiation which was not quantified. This highlights the importance of robust quantification of received dose in radiobiology studies (Pentreath, 2009).

3.2. *The effects of ionising radiation on the behaviour & histopathology of Crustacean species*

Ionising radiation has been demonstrated to induce behavioural changes in a number of crustacean species including crabs (Engel, 1967), prawns (Stalin et al., 2013a) and crayfish (Rodriguez and Kimeldorf, 1976). Alterations to behavioural patterns are fundamental in environmental risk assessments since these perturbations may arise as an adaptive mechanism to chronic contaminant exposure and have the potential to alter species–species interactions (Dell'Omo, 2002). The available literature regarding behavioural impacts of radiation involves mostly acute exposures to high doses of radiation (Engel, 1967; Rodriguez and Kimeldorf, 1976), with the magnitude of behavioural changes correlating with dose levels. For example, Engel (1967) assessed the impact of both chronic and acute radiation exposures on the behaviour of the blue crab, *Callinectes sapidus*, a highly aggressive and cannibalistic species (Bushmann, 1999). A reduction in aggressiveness of *Callinectes sapidus* specimens subject to single acute irradiations with $^{6°}$Co doses from 40 to 640 Gy was observed, whilst higher doses induced a catatonic state. Continuous exposures to lower doses (0.72, 1.64 & 6.53 Gy/d^{-1}) for 70 days induced cessation of feeding and abnormal behavioural patterns deviating from the normal pugnacious nature of *C. sapidus*, with the extent of behavioural effects relating to dose. Whilst the received dose remains significantly higher than estimates of the highest external doses in freshwater systems immediately after the Chernobyl accident ([4.2–8.3 mGy/hr^{-1} from bottom sediments] Kryshev et al., 2005), the finding that prolonged exposures may perturb behavioural patterns has implications for contaminated areas where radiation levels remain elevated over long time scales. Furthermore, limited data suggests induction of behavioural effects at lower, environmentally relevant doses. Stalin et al., (2013a) demonstrated behavioural changes including alterations to swimming patterns in the giant freshwater prawn, *Macrobrachium rosenbergii* at acute gamma doses of 3 mGy.

Few studies have considered the impacts of ionising radiation on morphological and histological parameters in crustaceans. Stalin et al., (2013a,b) demonstrated induction of histological and morphological aberrations including swollen and necrotic lamellae in the gill, deformations of the uropod, and discolouration of the abdomen in *M. rosenbergii* over a dose range of 3–3000 mGy (Stalin et al., 2013a), with the magnitude of effects relating to dose. Iwasaki (1973) adopted a histological approach to assess gamma radiation-induced effects in oogonia and oocytes of the brine shrimp, *Artemia salina*. A dose-dependent increase in cellular deformations and the number of pyknotic cells (cell degradation characterised by chromatin condensation) was recorded over a high dose range of 250–3000 Gy from Cobalt-60. Furthermore, Mothersill et al., (2001) recorded perturbations to cytoplasmic organelles in hematopoietic cultures of *Nephrops norvegicus* at gamma doses of 0.5 Gy. Deformations included abnormal mitochondrial-rough endoplasmic reticulum complexes at 0.5 Gy, progressing to complete disintegration of the cellular cytoplasm at doses of 5 Gy. Structural perturbations to the gill lamellae of crustaceans have been recorded in response to a number of toxicants (Li et al., 2007; Saravana Bhavan and Geraldine, 2000) and may ultimately impair gill functioning (Tamse et al., 1995) leading to asphyxia. Future studies should consider histological impacts on the crustacean gill using chronic, environmentally relevant radiation doses in order to corroborate this finding. A decrease in the hepatosomatic index of *M. rosenbergii* was also observed as a consequence of radiation exposure (Stalin et al., 2013b) which may provide further evidence that radiation elicits alterations to energy budgets since changes to the HSI may reflect mobilization and utilization of energy reserves (Sánchez-Paz et al., 2007).

Behavioural analysis of crustacean species exposed to ionising radiation has relied largely upon anecdotal visual observations over a defined time period (Stalin et al., 2013a). This approach is subject to a number of limitations including a lack of test standards (Kane et al., 2004), a low sensitivity comparable with video-based behavioural analysers and the potential for individual bias. Furthermore, the available studies have employed acute radiation exposures which may induce different behavioural effects to equivalent doses delivered over longer time scales (Solomon et al., 2009). Future studies should couple chronic, environmentally relevant exposure durations with a high-throughput behavioural tracking system. Such systems minimise bias by providing sensitive, reliable recordings of small animal behaviour under controlled conditions.

4. The effect of ionising radiation on reproduction in Crustaceans

Reproductive endpoints are frequently the subject of ecotoxicological and environmental risk assessments studies since perturbations to reproduction may impact upon the long-term survival of a species and hence alter ecosystem dynamics (Anderson and Wild., 1994; Dallas et al., 2012). A large number of publications have focused on radiation-induced effects on reproductive parameters in aquatic invertebrates, with the reported dose level at which significant effects occur varying by at least two orders of magnitude (Harrison and Anderson, 1996). This remarkable variability is exemplified within the crustacean subphylum; for example Alonzo et al., (2008a,b) recorded a delayed brood production in *D. magna* exposed to 15 mGy/hr^{-1} over a 23 day period (total dose of 0.345 Gy), however 10 Gy was needed to elicit a delay in the reproduction of the marine copepod, *Paracyclopina nana* (Won and Lee, 2014). Differences in the exposure duration, specific radionuclide and endpoint employed preclude development of a generalised 'dose limit' for reproductive effects in crustacean species. One of the priority areas for future research in radioecology is assessing the consequences of multigenerational radiation exposure. This was identified in the research agenda of the Strategy for Allied Radioecology (STAR) group (Hinton et al., 2013) on the basis that exposures across generations have long been a focus in human radiobiology and epidemiology studies (Dubrova et al., 2000; Koturbash et al., 2006; Nomura, 1988), but comparatively ignored in non-human biota. Radiation-induced perturbations to reproductive parameters may be particularly relevant in multigenerational exposure scenarios, since such perturbations may alter population dynamics and the subsequent ability of offspring to adapt to environmental stressors (Alonzo et al., 2008b; Lynch, 1989, 1992).

The available literature within the crustacean subphylum suggests the presence of effects over multiple generations (Alonzo et al., 2008a; Plaire et al., 2013; Massarin et al., 2010; Parisot et al., 2015; Sarapultseva and Gorski, 2013). Alonzo et al., (2008a) and Massarin et al., (2010) recorded an increase in the magnitude of deleterious effects across generations in *Daphnia magna* exposed to chronic alpha irradiation and chronic waterborne uranium exposure, respectively, with severe impacts to fitness and reproduction in individuals of the F_2 generation. In contrast, the multigenerational study of Parisot et al., (2015) reported a degree of recovery in F1 generation daphnids and a reduced radiosensitivity relative to the parental generation across both lethal and sub-lethal endpoints (mortality and fecundity, respectively). Differential radiosensitivity between developmental life stages has been widely recorded within the crustacean subphylum, demonstrated in copepods (Bardill et al., 1977) and *Artemia* species (Metalli et al., 1961). The authors (Parisot et al., 2015) hypothesised that a cumulative radiation dose may be necessary to elicit compensatory mechanisms such as DNA repair, accounting for the differential radiosensitivity between generations. Studies of radionuclide exposed daphnids using RAPD–PCR methods have indicated transmission of DNA damage from adult female daphnids to progeny across generations (Parisot et al., 2015; Plaire et al., 2013). This may be mediated by an epigenetic mechanism, as has been proposed for the transmission of effects in *D. magna* exposed to a range of chemical toxicants (Vandegehuchte et al., 2010) causing alterations to gene expression across generations. Further molecular studies are necessary to elucidate this mechanism. Given the increase in magnitude of reprotoxic and the associated deleterious effects on population dynamics (Alonzo et al., 2008a, 2008b) observed over generations, it is imperative that future studies continue to adopt a multigenerational approach as studies derived from single generation exposures may underestimate risk (Massarin et al., 2010).

Studies within the crustacean subphylum are heavily biased toward female reproductive success, with typical endpoints including production of new eggs (Won and Lee, 2014), hatchability of eggs (Iwasaki, 1964; Sellars et al., 2005) egg mass (Alonzo et al., 2006, 2008a) and time of hatching (Gilbin et al., 2008). Comparatively, radiation-induced effects on male fertility have been ignored. To the author's knowledge no study has directly recorded the impacts of ionising radiation on male fertility. Sperm are considered sensitive to the influence of xenobiotic stressors including ionising radiation (Fischbein et al., 1997; Lewis and Ford, 2012; Marques et al., 2014). This is attributed to their lack of inherent defence systems such as antioxidant enzymes and DNA repair comparable with other biological systems (Trapp et al., 2014). A number of studies have confirmed the sensitivity of sperm to anthropogenic radionuclides. Following the Chernobyl catastrophe an elevated incidence of sperm morphological abnormalities and perturbations to spermatogenesis was observed in liquidators exposed to doses of up to 0.25 Gy (Cheburakov and Cheburakova, 1992; Fischbein et al., 1997). Furthermore laboratory studies of plaice, *Pleuronectes platessa*, have demonstrated that chronic exposures to environmentally relevant low doses of gamma radiation (0.24 mGy/hr^{-1}) are sufficient to cause a significant reduction in sperm number in these organisms (Knowles, 1999). Experimental evidence in aquatic invertebrates has suggested that reductions in sperm numbers may have subsequent effects at higher levels of biological organisation. This is exemplified by Dunn et al., (2006), who recorded a 55% reduction in the size of freshwater amphipod (*Gammarus duebeni*) broods after mating with males displaying a sperm count reduction of 56% (Lewis and Ford, 2012). Coupling the ecological relevance of perturbations to sperm parameters with the known sensitivity of sperm, it is imperative that future studies within the Crustacea include these endpoints within radiobiological studies.

5. Radiation-induced mortality in Crustaceans

Despite the recent trend towards studies using chronic exposures of sub-lethal doses, the available literature of radiation effects in crustaceans remains dominated by mortality studies. This data is often used to calculate lethal dose (LD_{50}) values (Dallas et al., 2012) in order to derive hierarchies of radiosensitivity across taxonomic groups (Blaylock et al., 1996; Harrison and Anderson, 1996). LD_{50} values are traditionally used in ecotoxicological studies to determine the ecological risk to species (Stark et al., 2004) and have also been employed in order to determine no observed effect concentration (NOEC) values (Garnier-Laplace et al., 2006) to inform radioprotection and regulatory efforts. However, many studies have highlighted the greater sensitivity and ecological relevance of reproductive endpoints in radiation studies compared with measurements of adult mortality (Garnier-Laplace et al., 2006; Jones et al., 2003; UNSCEAR, 2008). This was further emphasised by Alonzo et al., (2008b) who found that an increase in individual mortality had a reduced effect on *D. magna* population growth relative to perturbations to two reproductive biomarkers (See Fig. 3). In contrast, Stark et al., (2004) recorded that within some species stress-induced individual mortality had a greater effect on intrinsic population increase than perturbations to reproductive capacity. Mortality has the potential to alter the age distribution, death rate and density of a population (UNSCEAR, 2008). Furthermore, the derivation of LD_{50} values is necessary to elucidate a given species sensitivity to radiation and to determine appropriate dose levels to employ in laboratory radiobiology studies. This serves to reiterate

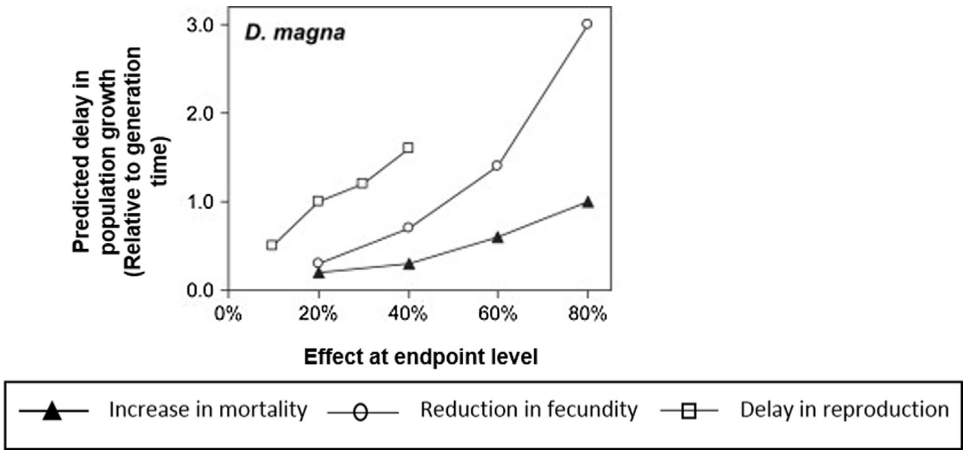

Fig. 3. Predicted delay in population growth relative to generation time in *Daphnia magna* populations exposed to alpha radiation determined using single generation models. The effects of increased mortality, fecundity reductions and delays in reproductive processes at the endpoint level are modelled. Reproduced with permission of Alonzo et al., (2008b).

the importance of including a number of endpoints in radioecology studies and that in some cases derivation of lethal dose data remains relevant.

Within the crustacean subphylum, the dose at which mortality occurs displays remarkable variability (Dallas et al., 2012). Recent work using the harpacticoid copepod *Tigriopus japonicus* demonstrated tolerance of external gamma radiation doses of up to 600 Gy, with mortality only occurring 5 days after cessation of exposure (Han et al., 2014). This is within the same order of magnitude as some bacteria and protozoan species, groups considered amongst the most radioresistant organisms (Copplestone et al., 2001). Furthermore, upon irradiation of dry egg masses, Iwasaki et al., (1971) demonstrated extreme radioresistance in *Artemia salina* nauplii of up to 2780 Gy one day after hatching. However, *Artemia* cysts display remarkable resistance to a range of stressors (MacRae, 2003) attributed to their greatly reduced metabolic and developmental activity prior to hatching and therefore are not considered representative of other crustacean species. Conversely, Rees (1962) reported a 30 day LD_{50} of ~15 Gy in the grass shrimp, *Palaemonetes pugio* which is within the upper bounds of radiosensitivity of some mammalian species (Blaylock et al., 1996). There are many problems associated with using lethal dose data to compare radiosensitivity across organisms. For example, there is a lack of standardisation of the duration used to calculate lethal dose data varying from 4 days (Han et al., 2014) to 40 days (Engel, 1973) which may greatly influence the final value. For chemical toxicants, this parameter has been standardised in published guidelines for tests using *Daphnia* species (OECD, 2004), enabling direct comparisons of LD_{50} values across stressors. Radiobiological studies would benefit from adopting a similar approach in order to aid comparative ability.

Comparatively, the effects of chronic radiation doses on mortality in crustaceans have been underrepresented. Marshall (1966) exposed 25 populations of *Daphnia pulex* to external gamma radiation over a 55 week period for 18.5 h a day with doses ranging from 0 to ~5.1 Gy/d^{-1}. At the three highest dose levels (~5.1 Gy/d^{-1}, ~4.8 Gy/d^{-1} and ~4.36 Gy/d^{-1}) populations crashed and became extinct, which the author attributed to an increase in individual death rate approaching the upper limit of the sustainable birth rate. Other monitored parameters such as the% of aborted eggs and embryos were shown to increase at dose levels below those leading to extinction, reiterating the greater sensitivity of reproductive endpoints comparable to mortality. Parisot et al., (2015) corroborated these findings, reporting a slight but non-statistically significant increase in mortality in ^{137}Cs exposed *D. magna* at a dose rate of 35.4 mGy/h^{-1}, with sub-lethal impacts occurring at much lower dose rates of 0.007 mGy h^{-1}. Engel (1967) exposed blue crabs, *Callinectes sapidus*, to acute doses (maxium of 180 min exposure) of gamma radiation from $^{6^\circ}$Co over a total dose range of 40–640 Gy at a dose rate of 219 Gy/hr^{-1}. The author also continually exposed *C. sapidus* to dose rates of 0.032, 0.073 and 0.29 Gy/hr^{-1} over a 70 day period. Following acute exposure a 30-day LD_{50} of 510 Gy was recorded. In contrast, crabs subjected to a total accumulated dose of ~460 Gy at the dose rate of 0.29 Gy/h^{-1} over a 70 day period displayed 100% mortality. Although the difference in total dose precludes useful comparison, the greater sensitivity of *C. sapidus* following continuous exposure underpins the importance of the exposure duration in determining biological impact in radiobiology studies. Dose rate has been reported to be an important factor in determining biological effects across a range of organisms including insects (Russell et al., 1958), humans (Elmore et al., 2006) and rodents (Russell et al., 1959). For example, Shimada et al., (2005) demonstrated a dose rate dependency of transgenerational mutation frequencies in spermatogonial stem cells of Medaka, *Oryzias latipes*. The authors exposed male medaka to an 80 TBq^{137} Cs source at dose rates of 3 Gy/min and 9500 Gy/min, and recorded a lower mutation frequency at each total dose (1.9, 3.2 and 4.75 Gy) in the 3 Gy/min group.

6. Conclusions

Despite numerous international directives and decades of research into the biological effects of radiation, significant gaps in our knowledge still remain. Although current research trends indicate an increase in the number of publications using environmentally relevant radiation sources and durations (Dallas et al., 2012), a significant disparity between the number of acute and chronic studies persists. This is exemplified within the FREDERICA radiation effects database. Within this database, 64% of the data points were obtained following acute radiation exposures, with 36% following chronic exposures. Furthermore, the available chronic data is heavily biased towards fish, mammals and terrestrial plants with a scarcity of data evident in the crustacean subphylum (See Table 1 (Copplestone et al., 2008). Another major limitation is the discrepancy between the available data for laboratory toxicity tests comparable with field studies. The majority of field data is heavily biased towards small mammals (Baker et al., 1996; Beresford et al., 2008; Chesser et al., 2000), fish (Dallas et al., 1998; Jonsson et al., 1999; Sugg et al., 1996 Jonsson et al., 1999; Sugg et al., 1996), plants (Kovalchuk et al., 1998, 2000; Syomov et al., 1992) and

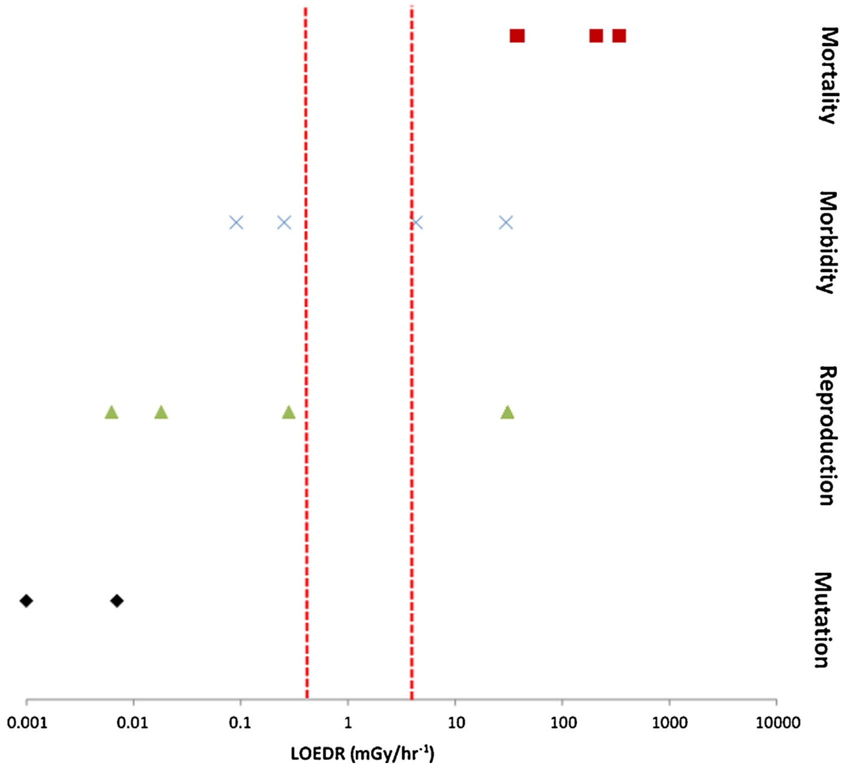

Fig. 4. Summation of the available chronic effect data within the crustacean subphylum across four endpoints; mortality, morbidity, reproduction and mutation. The lowest observed effect dose rate (mGy/h^{-1}) is used on a logarithmic scale. The band of dose rates selected by the ICRP (2008) DCRL for the reference crab is indicated by.

birds (Bonisoli-Alquati et al., 2010; Galván et al., 2014; Hermosell et al., 2013). Comparatively, the majority of field studies regarding crustacean communities exposed to radionuclides are focused on bioaccumulation of radioactive materials (Marzano et al., 2000) relating to trophic transfer, or calculating estimates of received doses (Murphy et al., 2011; Batlle et al., 2014).

A summary of the available chronic effect data and the corresponding lowest observed effect dose rate (LOEDR) in crustacean species is shown displayed in Fig. 4. From the limited available data, a tentative hierarchy of radiosensitivity in the four endpoints can be derived as follows: mutation > reproduction > morbidity > mortality. Whilst it must be reiterated that this is based on extremely limited data (two data points for mutation, see Fig. 4), this may challenge the assumption that reproduction is the most sensitive endpoint of radiation exposure in non-human biota (UNSCEAR, 2008). Responses at the molecular level, i.e., alterations to gene expression, are frequently recorded to be sensitive indicators of toxicant exposure across a range of organisms (See Section 2). Although recent crustacean radiobiology studies have demonstrated a shift towards use of molecular level endpoints (See Section 2 (Han et al., 2014b; Parisot et al., 2015; Won and Lee, 2014)), the use of 'ecotoxicogenomics' (the integration of genomic techniques in response to environmental toxicant exposure (Iguchi et al., 2007)) has been a focus of studies of other toxicants IE endocrine disruptors for almost a decade (Seo et al., 2006). The observed sensitivity of mutation as an endpoint observed in this paper highlights the need to exploit the advent of cheaper and more accessible molecular analyses in order to validate the usefulness of mutation as an endpoint and evaluate the potential of these techniques as tools in environmental radioprotection and radioecology. It is important to note that establishing linkages between gene expression analyses and endpoints of higher levels of biological organisation such as reproduction, survival and development remains a significant challenge in applying ecotoxicogenomics to ecological risk assessments (Miracle and Ankley, 2005). Furthermore, transcriptional changes do not necessarily elicit a biological effect within a given organism (Schirmer et al., 2010). Whilst this approach offers huge potential, until clear experimental links can be drawn between alterations to gene expression patterns and effects at higher levels of biological organisation, monitoring endpoints with clear ecological implications such as reproduction, development and growth remains important.

In conclusion, this review has summarized the available historic and current literature pertaining to radiation-induced effects in an ecologically relevant and model subphylum. Such effects are observed over a wide range of dose rates and exposure sources, and could conceivably have ecological consequences for biota chronically exposed to elevated levels of radionuclides. At present however there is limited population level data for aquatic organisms inhabiting chronically contaminated areas. Indeed, a study of the abundance and diversity of macroinvertebrate communities at eight Chernobyl affected lakes (Murphy et al., 2011) found no evidence of radionuclide contamination impacting the ecological status of the water body, and recorded the highest taxon richness at the most contaminated lake (estimated total external doses of 30.7 µGy/hr). Clearly, further studies at higher levels of biological organisation are necessary to elucidate the potential ecological consequences of the effects outlined in this review. Finally, this review has highlighted the persistent paucity of data across commonly used endpoints in the crustacean subphylum and identified key gaps in the literature to enhance research within the field. These data gaps must be addressed in order to enhance the efficacy of the subphylum Crustacea as a reference point for the optimisation and development of environmental radioprotection frameworks.

Conflict of interest

The authors declare no conflict of interest.

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Does Chernobyl-derived radiation impact the developmental stability of *Asellus aquaticus* 30 years on?

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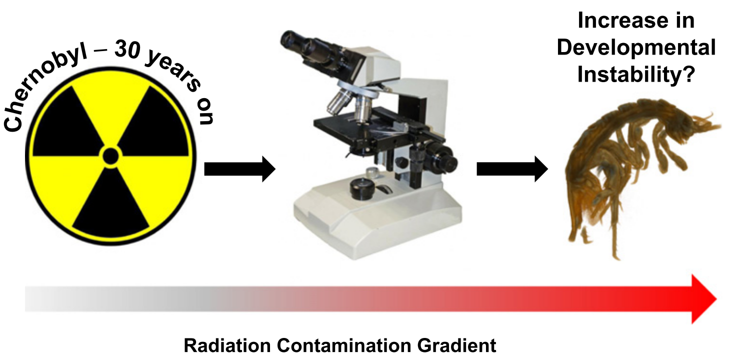
HIGHLIGHTS

- 30 year impacts of Chernobyl on development of *Asellus aquaticus* assessed
- Fluctuating asymmetry (FA) used as measure of developmental stability
- No increase in developmental stability along gradient of radioactive contamination
- Findings suggest resilience of aquatic invertebrate populations to radionuclides.
- Helps to understand the impacts of chronic exposures to radiation on ecosystems

GRAPHICAL ABSTRACT



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Fluctuating asymmetry
Aquatic invertebrate

ABSTRACT

Effects of long-term, environmentally relevant doses of radiation on biota remain unclear due to a lack of studies following chronic exposure in contaminated environments. The 1986 Chernobyl accident dispersed vast amounts of radioactivity into the environment which persists to date. Despite three decades of research, impacts of the incident on non-human organisms continues to be contested within the scientific literature. The present study assessed the impact of chronic radiation exposure from Chernobyl on the developmental stability of the model aquatic isopod, *Asellus aquaticus* using fluctuating asymmetry (FA) as an indicator. Fluctuating asymmetry, defined as random deviations from the expected perfect bilateral symmetry of an organism, has gained prominence as an indicator of developmental stability in ecotoxicology. Organisms were collected from six lakes along a gradient of radionuclide contamination in Belarus and the Ukraine. Calculated total dose rates ranged from 0.06–27.1 μGy/h. Fluctuating asymmetry was assessed in four meristic and one metrical trait. Significant differences in levels of pooled asymmetry were recorded between sample sites independent of sex and specific trait measured. However, there was no correlation of asymmetry with radiation doses, suggesting that differences in asymmetry were not attributed to radionuclide contamination and were driven by elevated asymmetry at a single site. No correlation between FA and measured environmental parameters suggested a biotic factor driving observed FA differences. This study appears to be the first to record no evident increase in developmental stability of biota from the Chernobyl region. These findings will aid in understanding the response of organisms to chronic pollutant exposure and the long term effects of large scale nuclear incidents such as Chernobyl and Fukushima.

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1. Introduction

The developmental stability of an organism is demonstrated by its capacity to produce an optimal phenotypic form under a specific set of environmental conditions (Palmer, 1994). Bilateral symmetry offers a method of determining levels of developmental stability founded on an a priori understanding of the ideal form; perfect bilateral symmetry. Fluctuating asymmetry (FA) refers to subtle, random deviations from the expected bilateral symmetry displaying a normal distribution with a mean of zero (Palmer and Strobeck, 1986). FA analyses have gained prominence as both an environmental monitoring tool and in evolutionary biology studies owing to the apparent ease with which such studies can be conducted and analysed compared with other phenotypic fitness indicators (Van Dongen, 2006). An increase in FA has been linked to a range of extrinsic and intrinsic stressors including organic pollutants (Jenssen et al., 2010), temperature (Vishalakshi and Singh, 2008a) and genetic stressors such as inbreeding (Özener, 2010). Studies have demonstrated relationships between levels of FA and traditional measures of fitness (Bakker et al., 2006; Silva et al., 2016), although the reliability of FA as a fitness indicator has been criticised (see Kruuk et al., 2003; Vishalakshi and Singh, 2008b).

The 1986 Chernobyl accident dispersed an estimated 5300 PBq of radioactivity into the environment (UNSCEAR, 2000) contaminating large areas of Europe. However, 30 years after the accident controversy still exists regarding the biological consequences of the incident and the dose levels at which these occur (Beresford and Copplestone, 2011; Beresford et al., 2016). For example, an order of magnitude decline in above ground invertebrates inhabiting Chernobyl (bumble bees, spiders, grasshoppers, butterflies and dragonflies) was recorded over a dose range of 0.1–10 μGy/h by Møller and Mousseau (2009) 20 years after the accident. Such dose rates fall within the range of exposure to terrestrial wildlife as a consequence of naturally occurring radionuclides (i.e. ^{40}K, ^{238}U and ^{232}Th) in the United Kingdom (Beresford et al., 2008). Conversely, no impact of radiation dose rates on the abundance and diversity of aquatic macroinvertebrates was found by Murphy et al. (2011) in Chernobyl contaminated lakes. The Tōhoku earthquake-tsunami at the Fukushima Dai-ichi nuclear power plant (NPP) in 2011 led to further contamination of the environment with radionuclides, with release estimates ranging from 7 to 50 and 1–5.5 PBq of ^{137}Cs for atmospheric and direct marine source term releases respectively (IAEA, 2015). At present, the radiological consequences of the Fukushima incident on non-human biota are unclear. These incidents underpin the necessity of robust evaluation of the impacts of radiation on wildlife.

Previous studies have demonstrated an increase in FA in populations of biota inhabiting areas impacted by elevated levels of radionuclides (Gileva and Nokhrin, 2001; Møller, 2002). For example, Oleksyk et al. (2004) demonstrated a 3.6 fold increase in mean skull FA in populations of the yellow necked mouse, *Apodemus flavicollis*, inhabiting areas closer to the failed Chernobyl reactor compared with reference populations over a range of dose rates from 0.107 to 4.146 μGy/h. Further, a high degree of FA was recorded in the freshwater mollusc, *Dreissena polymorpha* and floating pondweed *Potamogeton natans* from aquatic systems impacted by the Chernobyl incident (Yavnyuk et al., 2009). Williams et al. (2001) recorded an increase in morphological abnormalities in larval chironomids inhabiting Belarusian lakes impacted by the Chernobyl incident at ambient dose rates of 8–20 μSv/h, although FA was not directly quantified. FA therefore appears to be an appropriate indicator of radiation-induced developmental stress in organisms from the Chernobyl region.

This study aimed to assess the impacts of chronic radiation exposure along an established gradient of radionuclide contamination on the development of the water louse, *Asellus aquaticus* using FA as an indicator. FA has previously been induced in laboratory populations of *Asellus aquaticus* subjected to elevated temperatures (Savage and Hogarth, 1999) and is an effective indicator of developmental stability. *A. aquaticus* is a detritivorous isopod common in temperate freshwater ecosystems across Europe (Williams, 1962) that has gained prominence as an indicator species in ecotoxicity testing of sediment-borne contaminants (De Lange et al., 2005; McCahon and Pascoe, 1988).

2. Materials & methods

2.1. Sampling sites & collection of A. aquaticus

Six lakes were chosen ranging from 3 to 225 km in distance from the Chernobyl NPP. These sites exhibited varying degrees of contamination as a consequence of the Chernobyl incident (see Fig. 1). Where possible, localities with historic environmental data sets were selected. Samples were collected in littoral zones and amongst vegetation in June 2015 at three different sub sites of each lake by kick netting using a 1 mm mesh size net (EFE, UK). Following sieving, *A. aquaticus* were sorted and immediately preserved in 96% ethanol. Prior to analysis, samples were placed in randomly coded boxes to prevent measurement bias, a pervading problem in FA studies (Palmer, 1994).

2.2. Environmental parameters

Hydrochemical variable measurements, including conductivity oxygen saturation, pH and temperature were performed in situ using a multiparameter probe (HANNA Instruments 9828) at three stations of each lake. All lakes had similar fish communities comprised mainly of perch, roach and rudd (Smith et al., 2005; Murphy et al., 2011). Table 1 displays the measured environmental variables and available bathymetric data from Smith et al. (2005).

2.3. Estimation of external dose rates at sample sites

In the present study, external dose rates were calculated using deposition values of radiocaesium and strontium at sampling sites and dose conversion coefficients (DCC's) based on user inputted data for the geometry of *A. aquaticus* (Height = 2.2 mm, width = 1.7 mm, length = 4.7 mm and mass = 4.1 mg) using the ERICA tool (V1.2). DCC's for external dose rates were calculated to be 3.85×10^{-4} and 4.91×10^{-4} μGy/h per Bq/kg for ^{137}Cs and ^{90}Sr respectively.

Decay corrected activity concentrations of ^{137}Cs and ^{90}Sr in sediments were first calculated (Bq kg^{-1} fresh weight) as:

$$C_{sediment} = \frac{(D_{total})\ x\ (e^{-\lambda_r t})}{P_{sediment}\ x\ d_{sediment}}$$

Where $C_{sediment}$ = Fresh weight activity concentration of sediment Bq kg D_{total} = Total deposition of radionuclide in Bq m^{-2}λ_r = Decay constant for radionuclide in d^{-1}t = time in days $P_{sedimentl}$ = wet sediment bulk density in kg m^{-3}$D_{sediment}$ = Depth of sediment in m within which the radionuclide has become mixed.

The dose estimates were based on a wet sediment bulk density of 1300 kg m^{-3} and assuming mixing to a depth of 0.15 m (Smith et al., 2005). The organism was assumed to occupy the sediment-water interface. Strontium – 90 (^{90}Sr) and radiocaesium (^{137}Cs) are the two major contributors to environmental radiation doses received by biota over chronic time scales (IAEA, 1995). Sr-90 is associated with fuel particles and shows a rapid decline with distance from Chernobyl relative to radiocaesium (Mück et al., 2002). Consequently, total doses from Strontium were only considered at Yanovsky Crawl and Glubokoye lake (3 and 10 km from the Chernobyl NPP respectively) as concentrations at other water bodies are known to be insignificant (Murphy et al., 2011) in comparison with radiocaesium. A generic value of 0.06 μGy/h was added to estimate cumulative external radiation doses in study lakes to account for natural background radiation (Murphy et al., 2011).

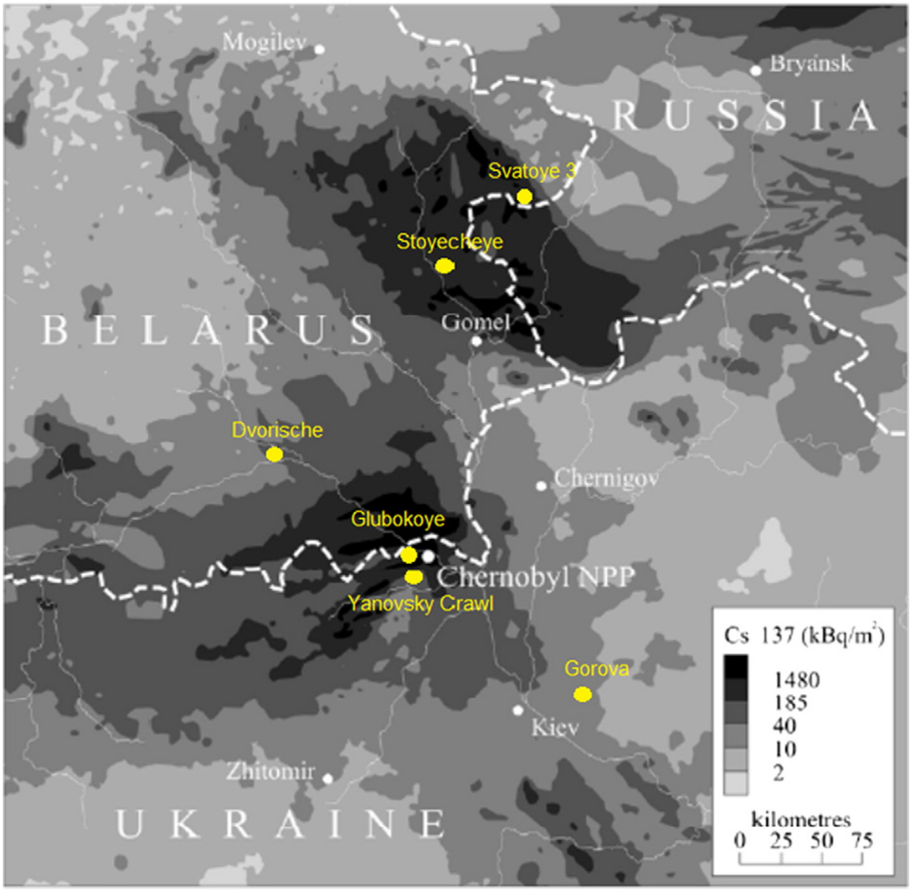

Fig. 1. Map indicating the six sample sites for *A. aquaticus*. Radiocaesium deposition is indicated. Reproduced from Smith and Beresford (2005) with permission.

2.4. Estimation of internal dose rates at sample sites

Internal dose rates were calculated based on average measurements of ^{137}Cs and ^{90}Sr in lakes at various depths (see Table 3). Dose estimates were made using the ERICA tool (V 1.2) and DCC's for internal beta-gamma radiation based on user-defined geometry (see Section 2.3). DCC values of 1.9×10^{-4} and 6.3×10^{-4} μGy/h per Bq/kg were calculated for ^{137}Cs and ^{90}Sr respectively. Since no data exists for activity concentrations of ^{137}Cs and ^{90}Sr in the least contaminated lake, Gorova, a value of 0.063 μGy/h was added to account for background exposure following Kryshev and Sazykina (1995).

2.5. Calculation of fluctuating asymmetry

Four traits were selected for analysis of FA in *A. aquaticus*; first antennae length, propodos and merus width of the first paraeopod and carpus width of the second paraeopod. Selection of these traits was based on previous studies using similar characters to demonstrate a relationship between environmental stress and FA in *Asellus aquaticus* (e.g. Savage and Hogarth, 1999). A preliminary study (Data not shown) further demonstrated that the selected traits were robust, easily quantifiable and damage during handling would not bias measurements. As an additional measure, the number of segments of the first antennae was quantified following three replicate counts. Asymmetry in segment number has been previously demonstrated in *A. aquaticus* and appears to be linked to antennal length asymmetry (Savage and Hogarth, 1999). A total of 3988 measurements were conducted on 394 organisms across the six lakes. Organisms were sexed by analysis of the pleopods and measurements of body length conducted following Bertin et al. (2002). Characters were dissected, mounted on slides and photographed using a Leica DFC310 camera following flattening under a 22 × 40 mm coverslip. Two independent blind measurements were taken on each trait using ImageJ (v 1.48). All measurements were conducted by one researcher (NF) over a two week period to minimise inter-observer variability (Lee, 1990). FA was calculated using the FA1 index as: FA = Mean |R-L|, where R and L represent the right and left side trait measurements in μm respectively. For comparisons of mean pooled FA values across

Table 1
Physical and chemical characteristics of the six study lakes. ND = No Data.

Lake	Conductivity (μS/cm)	Oxygen saturation (%)	pH	Temperature (°C)	Max depth (m)[a]	Surface area (km^2)[a]	Distance from Chernobyl (km)
Svyatoye-3	122.1	81.6	7.8	23.00	2.9	0.250	225
Stoyacheye	230	89.4	8.7	24.05	17.0	0.460	157
Dvorsiche	200	68.9	7.82	23.73	3.5	0.128	95
Glubokoye	184.8	66.3	7.92	24.92	7.3	0.100	10
Yanovsky Crawl	275	108.5	9.4	23.29	ND	ND	3
Gorova	178.5	185	9.69	22.17	ND	ND	125

[a] Data from Smith et al. (2005).

Table 2
Estimated activity concentrations of sediment and concomitant external doses received by *Asellus aquaticus* based on radiocaesium and strontium deposition at six localities in Belarus and Ukraine. Data from Smith et al. (2005) unless otherwise stated.

Lake	^{137}Cs deposition at site (kBq m^{-2})	^{137}Cs activity of sediment (Bq kg^{-1})	External dose rate ^{137}Cs (μGy/h)	^{90}Sr deposition at site (kBq m^{-2})	^{90}Sr activity of sediment (Bq kg^{-1})	External dose rate ^{90}Sr (μGy/h)	Total external dose rate (μGy/h)
Glubokoye	15500[a]	40,706	7.84	12,000	30,526	7.51	15.35
Yanovsky Crawl	14800[b]	38,867	7.5	16,300	41465[a]	10.2	17.7
Svyatoye 3	1748	4591	0.886	ND	N/A	N/A	0.886
Stoyacheye	288	756	0.145	ND	N/A	N/A	0.145
Dvorische	100[c]	262[d]	0.0513	ND	N/A	N/A	0.0513
Gorova	10	26	1.93E−03	ND	N/A	N/A	1.93E−03

[a] Data from Sansone and Voitsekhovitch (1996).
[b] Data from Nazarov and Gudkov (2009).
[c] De Cort et al. (1998).
[d] Data from Ukrainian Hydrometerological Institute, Kiev.

sites, the size-corrected FA2 index was employed: $FA2 = \text{mean}\ [|R - L| / (R + L) / 2)]$ to account for differences in trait size as suggested in Palmer (1994).

2.6. Statistical analyses

A two-way mixed model ANOVA was performed on asymmetry data for each trait x lake combination, with individual as a random factor and orientation as a fixed factor. This procedure tests for the significance of all between-sides variation relative to measurement error (ME) (Palmer, 1994). This method also tests significance of directional asymmetry (DA), a pattern of variation wherein one side is consistently larger than the other, relative to FA simultaneously. Additional one-sample t-tests were performed against a mean of zero to further test for the presence of DA (Baker and Hoelzel, 2013). The aforementioned procedures cannot deduce whether the observed pattern of variation is due to true FA or asymmetry. Asymmetry refers to a pattern of R-L variation characterised by a bimodal distribution either side of zero (Palmer and Strobeck, 1986). Tests for asymmetry were performed using conventional kurtosis statistics and one-sample Kolmogorov-Smirnov tests. Finally the presence of size dependence, the association between organism size and magnitude of FA, must be accounted for as it can confound observed differences in FA between sites if different size ranges are present. Non-parametric tests of association are preferred for such analysis since they do no assume homogeneity of variance (Palmer and Strobeck, 2003). Spearman's rank-order correlation coefficients were therefore used to test the degree of size dependence of each trait with *A. aquaticus* body length.

Differences in levels of FA between sites were tested using multiple approaches. Firstly, a one-way ANOVA with Bonferroni post hoc tests was performed on size corrected FA data to analyse FA differences amongst lake populations. A general linear model (GLM) was then applied where site and sex were fixed factors and trait a random factor to ascertain the influence of sex on observed FA values. Mean asymmetry values were calculated by averaging replicate measurements to minimise the impact of measurement error (Arnqvist and Martensson, 1998). Owing to the non-normal distribution of the metrical segment asymmetry data, nonparametric analysis of variance (Kruskal-Wallis H Test) was used to analyse asymmetry differences between sites. A Spearman's rank-order correlation was used to assess the relationship between percentage of individuals exhibiting segment asymmetry and total dose rate.

The relationship between asymmetry and measured environmental parameters was analysed using regression analysis on log-transformed data. Bathymetric features such as lake area and maximum lake depth were considered to be time invariant and thus were not included in the analysis.

3. Results

3.1. Estimated radiation doses received by biota

Tables 2 and 3 display the estimated external and internal radiation doses at each of the sampling sites based on ^{137}Cs and ^{90}Sr activities in sediment and water. Maximum and minimum total doses were calculated at Glubokoye lake (27.1 μGy/h) and Gorova respectively (0.06 μGy/h, see Table 3).

Dose rate estimates based on deposition of radionuclides at study sites are subject to a significant degree of uncertainty, owing to the necessary simplifications and assumptions made to calculate these values (see Murphy et al., 2011). However, direct measurements of external radiation doses at Svyatoye-3 by Pungkun (2012) using a thermoluminescent dosimeter (TLD) array provided good agreement with dose estimates in the present study (0.8–1.8 μGy/h for measured average sediment concentrations), suggesting estimates in the present study provide an adequate assessment of radiation doses received by biota.

3.2. Departures from normality and measurement error

Conventional kurtosis statistics of each trait × lake combination are shown displayed below in Table 4. The majority of lake × trait combinations demonstrated slight leptokurtosis which is not attributed to antisymmetry. Where platykurtic distributions existed, Kolmogorov-Smirnov tests and visual inspections of FA frequency distributions

Table 3
Estimated internal and total doses received by *Asellus aquaticus* at six lakes along a contamination gradient in Belarus and the Ukraine based on activity concentrations of ^{137}Cs and ^{90}Sr in water and sediment. ^{137}Cs and ^{90}Sr in water based on average measurements at different depths in 2003.

Lake	^{137}Cs in water (Bq l^{-1})	Internal dose ^{137}Cs (μGy/h)	^{90} Sr in water (Bq l^{-1})	Internal dose ^{90} Sr (μGy/h)	Total internal dose (μGy/h)	Total dose rate (μGy/h)
Glubokoye	7.57	1.16	77.7	10.5	11.66	27.1
Yanovsky Crawl	2.2	0.336	18.7[a]	2.53	2.866	20.6
Svatoye #3	7.8	1.19	N/A	N/A	1.19	2.2
Stoyacheye	4.24	0.647	N/A	N/A	0.647	0.872
Dvorische	4.29	0.655	N/A	N/A	0.655	0.786
Gorova	ND	0.063	N/A	N/A	0.063	0.064

[a] Data based on average measurements from 1997 to 2008 from Nazarov and Gudkov (2009).

Table 4
Kurtosis statistics of each lake × trait combination.

	Gorova	Dvorische	Stoyacheye	Svyatoye-3	Yanovsky Crawl	Glubokoye
First antennae	−0.113	1.348	0.963	2.743	3.794	2.456
Propodos width	1.974	0.702	0.278	1.851	1.173	3.151
Merus width	2.923	0.035	−0.242	−0.642	0.824	0.879
Carpus width	0.128	0.507	0.087	0.641	0.807	0.763

revealed no significant differences from normality ($p > 0.05$). Analysis of the metrical dataset (number of antennal segments) revealed a leptokurtic distribution (high, narrow peak) which was significantly different from the normal distribution (Kolmogorov–Smirnov test, $p < 0.05$). This was attributed to the low range of observed R-L values in the data and is frequently observed in metrical traits (i.e. pectoral fin rays in fishes [Johnson et al., 2004; Young et al., 2009]). Since antisymmetry is characterised by a platykurtic distribution (Palmer and Strobeck, 1986), and segment asymmetry did not significantly differ from 0, antisymmetry was not considered.

3.3. Size dependence

No correlation between body length of asellids and magnitude of asymmetry (Spearman's rank order correlation coefficient, $p > 0.05$) was recorded indicating that size dependence was not evident in the present study.

3.3.1. Fluctuating asymmetry

Significant differences in FA were recorded amongst sample sites ($p < 0.05$, see Table 5) indicating differences in developmental stability between localities (see Table 5).

A post-hoc Bonferroni test revealed significant differences in FA values between Svyatoye-3 and all sites excluding Dvorische and Glubokoye lake (see Fig. 2) when information from multiple traits was considered ($p \leq 0.05$). At Svyatoye-3, mean pooled asymmetry of four traits was 0.063 ($n = 228$), significantly greater than Yanovsky Crawl (0.043, $n = 274$), Stoyacheye (0.032, $n = 271$) and Gorova (0.045, $n = 277$). First antennae length exhibited the greatest degree of asymmetry overall (0.052, $n = 398$), however differences between trait were not significant ($p > 0.05$). Lowest mean asymmetry values were recorded for the carpus of the second paraeopod (0.044, $n = 394$). The general linear model revealed no significant differences in asymmetry between sexes, independent of locality or specific trait measured ($p > 0.05$, see Table 5).

3.3.2. Fluctuating asymmetry in segment number

No significant differences in the magnitude of segment asymmetry (calculated as |R-L|) were recorded between sampling sites (Kruskal-Wallis H, $p > 0.05$). Maximum right-left differences of 4 segments were displayed at lakes Glubokoye and Svyatoye-3. The highest % of individuals exhibiting segment asymmetry was Svyatoye-3, wherein 35.6% of asellids exhibited asymmetry in segment number. The lowest number of individuals displaying segment asymmetry (21.7%) was recorded at Gorova. Antennal segment asymmetry and total dose rate approached a positive relationship ($r^2 = 0.51$), however this relationship was non-significant (Spearman's rank-order correlation, $p > 0.05$). The raw data showed that in 72% of cases, asymmetry of segment number and antennal length was in the same direction, suggesting that antennal asymmetry in length and number of segments is linked as originally proposed in Savage and Hogarth (1999).

3.3.3. Fluctuating asymmetry in relation to environmental parameters

Regression analysis found no significant relationship between pooled FA and any of the measured environmental parameters including conductivity ($R^2 = 0.063$, $p = 0.330$, $df = 1$, $F = 1.015$), temperature ($R^2 = 0.012$, $p = 0.663$, $df = 1$, $F = 0.198$), pH ($R^2 = 0.067$, $p = 0.301$, $df = 1$, $F = 1.141$) and dissolved oxygen levels ($R^2 = 0.017$, $p = 0.603$, $df = 1$, $F = 0.281$).

3.3.4. Fluctuating asymmetry in relation to total radiation dose

Mean asymmetry at the most highly contaminated lake, Glubokoye (total dose rate of 27.1 μGy/h) was 0.053, 1.2 times greater than individuals from the site of lowest contamination, Gorova (total dose rate of 0.06 μGy/h) although these differences were non-significant (Bonferroni, $p > 0.05$). Mean asymmetry at Yanovsky crawl, with total dose rates of 20.6 μGy/h, was 0.043, within the range of sites exhibiting dose rates up to two orders of magnitude lower (i.e. Gorova). The relationship between asymmetry and radiation dose rate along the contamination gradient is shown in Fig. 3. The value for the regression slope was 0.008 (see Fig. 3), demonstrating the lack of relationship between the two variables which was non-significant ($p > 0.05$).

4. Discussion

The present study aimed to determine the impact of Chernobyl-derived radionuclides on the developmental stability of the aquatic invertebrate, *Asellus aquaticus*, using fluctuating asymmetry as an indicator. Six localities were sampled encompassing the full range of radiocaesium contamination across both Belarus and Ukraine. No relationship between radiation dose rate and pooled asymmetry of four morphometric traits was found. Asymmetry in segment number of the first antennae approached a positive relationship with radiation dose rate; however this was non-significant (see Section 3.4.2).

These findings are different to those in the available literature which suggest an increase in FA in response to radiation exposure. This has been documented for a range of biota including fish (Lajus et al., 2014; Zakharov et al., 1996), small mammals (Gileva and Nokhrin, 2001; Oleksyk et al., 2004), birds (Møller, 1993), insects (Møller, 2002) and plants (Møller, 1998). Studies of aquatic invertebrates from the Chernobyl region further support an increase in FA as a result of radionuclide exposure. Yavnyuk et al. (2009) measured FA in two species, the zebra mussel *Dreisenna polymorpha* and floating pondweed, *Potamogeton natans*. The authors recorded a 25-fold increase in asymmetry of *D.*

Table 5
Results of the general linear model analysis with a mixed model ANOVA performed on size corrected asymmetry data in *A. aquaticus*. Site and sex were fixed effects, trait a random effect.

Source of variation	Mean square	F value	Df	p value	Interpretation
Site	0.014	3.238	5	0.035	FA varies between localities
Sex	0.000	0.704	1	0.803	FA does not differ between sexes
Trait	0.006	1.513	3	0.396	FA does not vary dependent on trait
Site ∗ sex	0.001	0.309	5	0.900	FA differences between sexes not dependent on site
Site ∗ trait	0.004	2.383	15	0.052	FA differences between sites not dependent on trait
Sex ∗ trait	0.003	1.402	3	0.280	FA difference between traits not dependent on sex

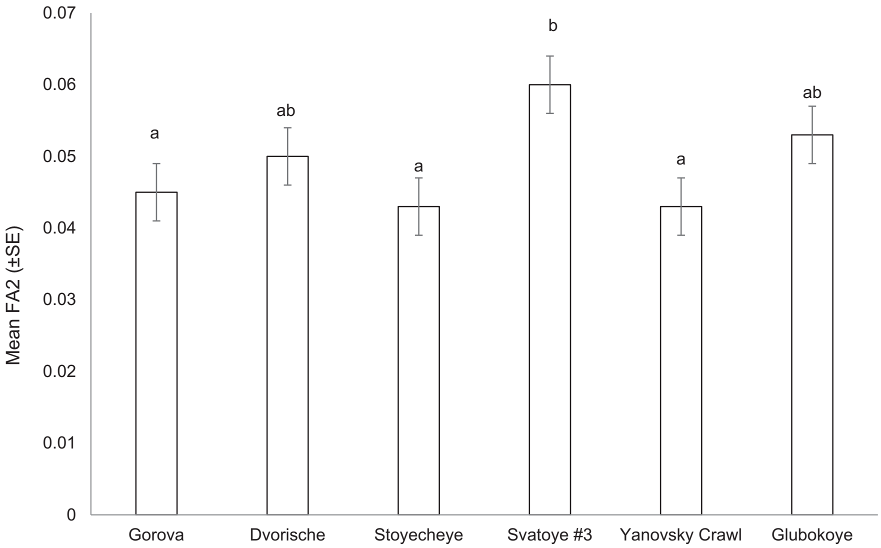

Fig. 2. Fluctuating asymmetry values (mean ± SE; $FA = |R - L| / ((R + L) / 2)$) pooled from four traits (first antennae length, propodos and merus width of the first paraeopod, and carpus width) in *Asellus aquaticus* collected from six lakes along a gradient of radionuclide contamination in Belarus and Ukraine. Sites are plotted in order of increasing contamination from left to right. Different letters denote significant differences ($p < 0.05$) between sites, matching letters signify no significant difference.

polymorpha collected from the Chernobyl cooling pond compared to reference areas within the Dnieper river. A smaller increase in *P. natans* FA of 1.4-fold compared to samples collected from the Kiev reservoir was documented. Molluscs have been demonstrated to accumulate and retain radionuclides at concentrations orders of magnitude higher than other aquatic biota (Frantsevich et al., 1996; Jaeschke et al., 2015) and are susceptible to elevated dose rates. For example, Gudkov et al. (2016) recorded dose rates of 350–420 μGy/h in the freshwater snail, *Lymnaea stagnalis* collected from Glubokoye lake over a study period from 1998 to 2014. Dose rate estimates to *Asellus aquaticus* in the present study for Glubokoye lake were 27.1 μGy/h, an order of magnitude lower. The lower dose rates received by *A. aquaticus* in the present study may not have been to sufficient to cause a discernible increase in developmental instability as indicated by FA.

Some studies have proposed exposure thresholds below which no discernible increase in FA above background level is expected. For example, Oleksyk et al. (2004) found significant correlations between intramuscular ^{137}Cs concentrations and right-left differences in the yellow-necked mouse, *Apodemus flavicollis* collected from around the Chernobyl exclusion zone. The authors suggested a dose range for internal exposure of 0.132–0.297 μGy/h above which FA may increase significantly above background level. Five of the six sampled sites (See Table 3) in the present study had total internal dose rates exceeding this threshold, with no discernible increase in FA. This may be owing to inter-species differences in response to elevated doses of radiation. *Asellus aquaticus* has been shown to be resistant to a range of toxicants and capable of life history adaption to favour habitation of polluted environments (Fraser, 1980; Maltby, 1991). For example, Maltby (1991) demonstrated adaptive modification of reproductive investment toward fewer, large offspring in *A. aquaticus* inhabiting chronically contaminated sites as compared to a clean site. Although radiosensitivity varies greatly within taxa (Harrison and Anderson, 1996), it is generally accepted that radiosensitivity increases with the degree of biological complexity (Copplestone et al., 2001). Organisms such as birds, trees and mammals are believed to be more radiosensitive than invertebrates (Hinton et al., 2007). Consequently, dose thresholds for induction of FA

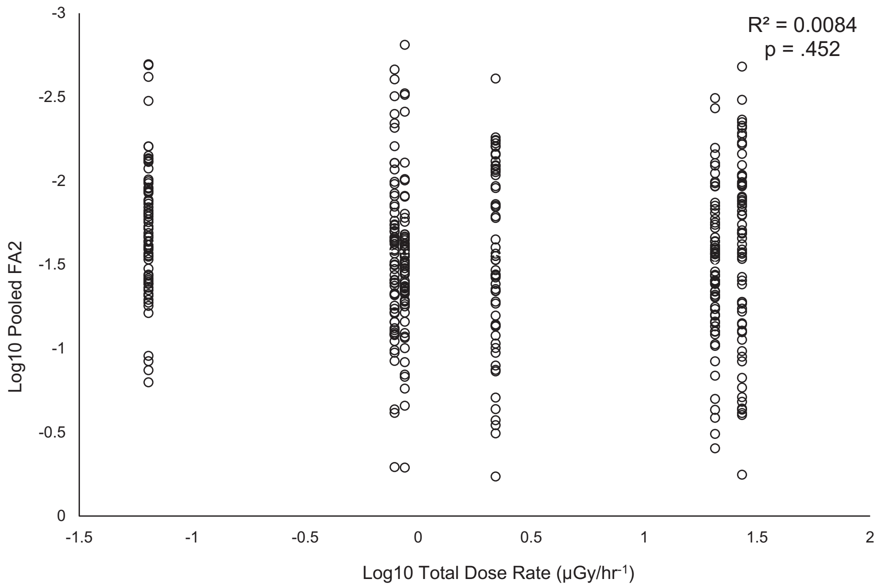

Fig. 3. Scatterplot of total dose rate in relation to pooled asymmetry of four morphometric traits (calculated as $FA = |R-L|/((R + L)/2)$) in *Asellus aquaticus* from the Chernobyl region.

may be significantly lower in vertebrate organisms and studies of FA may not be directly comparable.

Fluctuating asymmetry has frequently been reported to have low heritability (Kruuk et al., 2003; Leamy and Klingenberg, 2005; Woods et al., 1998) which is advantageous in its use as an environmental monitoring tool (Palmer and Strobeck, 1986) since observed patterns are expected to reflect stress within the population rather than its genetic structure. Therefore the lack of increase in FA recorded in the present study suggests that current dose rates in the Chernobyl region are insufficient to cause an increase in developmental instability in *A. aquaticus*. Assuming *A. aquaticus* to be univoltine (Brattey, 1986), populations have undergone almost thirty generations since the Chernobyl incident. It is therefore possible that organisms may have adapted to chronic radiation stress. Studies have demonstrated adaptation of aquatic invertebrate populations exposed to a range of pollutants over relatively short timeframes (Goussen et al., 2015; Hochmuth et al., 2015; Sun et al., 2014). For example, Sun et al. (2014) found evidence for adaptation to tributytltin oxide (TBTO) in the sexually reproducing marine copepod, *Tigriopus californicus* in seven generations. Elevated mutation frequencies have been reported in a range of organisms from Chernobyl (see Møller & Moussea, 2015 for meta-analysis). However, studies of potential adaption to chronic radiation stress are limited and at present inconclusive (i.e. Geras'kin et al., 2011; Klubicová et al., 2010). Future work should focus on the application of whole genome techniques to study mutation rates and potential adaptation to chronic radiation stress in Chernobyl biota.

In the present study, first antennae length exhibited the greatest degree of asymmetry (see Section 3.4). In many crustacean species, antennae are considered a secondary sexual character involved in the detection and location of receptive females (Dunn, 1998; Lefebvre et al., 2000). In *A. aquaticus*, first antennae possess aesthetascs (Wägele, 1983) which are believed to have a chemosensory function in mate detection, supporting the role of this trait as a secondary sexual character. Møller and Pomiankowski (1993) argue that patterns of FA within secondary sexual characters differ from typical morphological traits and are often significantly greater in the former. This is thought to be due to production costs in developing elaborate secondary sexual characters and the strong associated directional selection which may select against modifiers that buffer development (Manning and Chamberlain, 1994). The elevated FA in first antennae in the present study supports an increase in FA in secondary sexual characters relative to typical morphological traits.

Significantly greater mean asymmetry was observed at Svyatoye-3 relative to all sites excluding Dvorische and Glubokoye lake when data from multiple traits was considered. Differences in asymmetry were not found to be related to any of the measured environmental parameters using regression analysis (see Section 3.4.2). Elevated developmental instability at this site may be due to a biotic stressor, for example an increased parasitic burden. A number of studies have demonstrated positive correlations between parasite burden and FA (i.e. Alibert et al., 2002; Bonn et al., 1996; Cuevas-Reyes et al., 2011). *Asellus aquaticus* is an intermediate host to acanthocephalan parasites (Brattey, 1986) and has also been demonstrated to harbour *Wolbachia*, a parasitic intracellular bacteria (Bouchon et al., 1998). Acanthocephalan infection has been linked to an increase in FA in another freshwater aquatic invertebrate species, *Gammarus pulex*, which occupies an overlapping ecological niche (Alibert et al., 2002; Graça et al., 1994). Furthermore, sex ratios of *A. aquaticus* at Svyatoye-3 were skewed in the favour of females relative to other sites (0.31:0.69 males to females, Data not shown), which could be indicative of *Wolbachia* infection, since this parasite typically causes feminization of host organisms (Rigaud et al., 1999). Future research will aim to quantify the prevalence of *Wolbachia* infection in Belarusian and Ukrainian populations of *A. aquaticus* using a PCR-based approach.

5. Conclusions

No relationship between total radiation dose rates and magnitude of FA was recorded in the present study. The percentage of individuals exhibiting segment asymmetry approached a positive relationship with total dose rate; however, this was not found to be statistically significant. Significantly elevated FA was recorded at a single site, and was not attributed to radiation dose or any of the measured environmental parameters, suggesting a biotic stressor driving an increase in developmental instability. Whilst Lajus et al. (2014) recorded only a minor impact of radiation on FA in two fish species, this finding appears to be novel in the literature in finding no discernible impact of radiation on FA in biota from the Chernobyl region. The present study utilized the full range of contamination in aquatic systems from within the highly contaminated 10 km zone to 225 km in distance from the NPP (see Fig. 1), providing a comprehensive assessment of the potential effects of Chernobyl-derived radiation on FA in *A. aquaticus.* In contrast, many of the previous studies of FA in Chernobyl biota utilized only a single contaminated and reference site. For example, Møller (2002) and Lajus et al. (2014) studied FA in stag beetles and fish respectively from only a single contaminated and reference area. The greater range of dose rates in the present study strengthens any observed associations between radionuclide contamination and FA and may account for the differences between the present and previous studies of developmental instability in Chernobyl biota.

The significance of the Chernobyl incident for both members of the public and policy makers presents challenges for the scientific community, as described by Chesser and Baker (2006). It is imperative that negative results are regarded in the same manner as those reporting positive results. Recent studies (i.e. Deryabina et al., 2015; Webster et al., 2016) have recorded abundant wildlife populations in the Chernobyl exclusion zone and challenge perceptions of the detrimental impacts of radiation exposure. Clearly, the effects of radiation on non-human organisms are not ubiquitous and likely depend on dose rate, species and effect endpoint. Further robust studies of individual level effects are necessary. The lack of increase in FA in the present study challenges previous findings of the impacts of chronic radiation exposure on the development of organisms. Such findings will aid in developing risk assessments of the impact of chronic radiation exposure on biota and will help elucidate the long term impacts of large scale nuclear incidents such as Chernobyl and Fukushima.

Conflict of interest

The authors declare no conflict of interest.

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Reproduction in the freshwater crustacean *Asellus aquaticus* along a gradient of radionuclide contamination at Chernobyl

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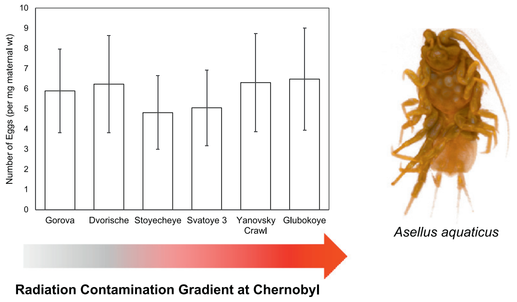
HIGHLIGHTS

- We assessed effects of Chernobyl radiation on crustacean reproduction.
- Fecundity of *Asellus aquaticus* assessed at dose rates from 0.06–27.1 μGy/h.
- No association of radiation with reproductive endpoints in *A. aquaticus.*
- Findings support proposed benchmarks for the protection of aquatic populations.
- Data can assist in management of radioactively contaminated environments.

GRAPHICAL ABSTRACT

- **No effects observed on reproduction of *Asellus aquaticus* along a gradient of radionuclide contamination**



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ABSTRACT

Nuclear accidents such as Chernobyl and Fukushima have led to contamination of the environment that will persist for many years. The consequences of chronic low-dose radiation exposure for non-human organisms inhabiting contaminated environments remain unclear. In radioecology, crustaceans are important model organisms for the development of environmental radioprotection. Previous laboratory studies have demonstrated deleterious effects of radiation exposure on crustacean reproduction. However, no studies have documented the effects of chronic radiation exposure on the reproduction of natural crustacean populations. Based on data from laboratory exposures, we hypothesised that populations of the freshwater isopod *Asellus aquaticus* exposed to radiation for thirty years at Chernobyl would display reduced reproductive output and altered timing of reproduction. To test this hypothesis, *A. aquaticus* was collected from six lakes at Chernobyl over two years with total dose rates ranging from 0.06–27.1 μGy/h. No significant differences in the fecundity, mass of broods or proportion of reproducing female *A. aquaticus* were recorded. Significant differences in the body mass of gravid females were recorded suggesting different timings of reproduction, however this was not related to radiation contamination. No significant effect of a range of environmental parameters on *A. aquaticus* reproduction was recorded. Our data suggests current dose rates at Chernobyl are not causing discernible effects on the reproductive output of *A. aquaticus*. This study is the first to assess the effects of chronic low-dose radiation exposure on the reproductive output of an aquatic invertebrate at Chernobyl. These findings are consistent with proposed radiological protection benchmarks for the maintenance of wildlife populations and will assist in management of environments impacted by radiation.

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1. Introduction

The accident on 26th April 1986 at the Chernobyl nuclear power plant (CNPP) led to the release of an estimated 5300 PBq (5.3×10^{17} Bq) of radioactivity into the environment (UNSCEAR, 2000). Vast areas of the former Soviet Union (fSU) and Western Europe were contaminated with radioactive substances, leading to radiation exposure of human and non-human organisms. A total of 220,000 people across Belarus, Russia and the Ukraine were permanently evacuated and a 30 km exclusion zone was established around the CNPP (UNSCEAR, 2000). Radioactivity in the aquatic environment was of concern owing to the proximity of the CNPP to the Pripyat river system and potential for contamination of the Kiev reservoir. Whilst dose rates decreased significantly in subsequent years owing to decay of short lived radionuclides and settling of radioactive substances to sediments, a number of 'closed' lake systems around Chernobyl retained relatively high levels of radioactivity. Such systems are typified by a lack of significant inflow or outflows of water, and are widespread in the Pripyat flood plain area and fSU countries affected by the Chernobyl accident (Smith and Beresford, 2005). The 2011 accident at the Fukushima Daiichi Nuclear Power Plant (FDNPP) caused by the Great East Japan Earthquake further contaminated a range of aquatic environments with radionuclides (IAEA, 2015), including a range of freshwater irrigation ponds and closed lakes. Understanding the effects of chronic radiation exposure on the aquatic environment is therefore a highly pertinent issue.

Immediately after the Chernobyl accident, wildlife inhabiting the exclusion zone were subjected to high doses of radiation with significant effects recorded for a range of organisms (See Smith and Beresford, 2005; Hinton et al., 2007). The chronic effects of Chernobyl-derived radiation on the environment are much less clear and remain highly controversial within the scientific community. No influence of radioactive contamination was observed on the abundance and diversity of macroinvertebrate species in Chernobyl affected lakes 18 years post-accident (Murphy et al., 2011). Recent studies of mammal communities in Chernobyl-affected areas (e.g Deryabina et al., 2015; Webster et al., 2016) have reported abundant mammal populations irrespective of the potential for radiation effects at the individual level. Conversely, population-level declines in the abundance of birds (Galván et al., 2011; Møller and Mousseau, 2007) and mammals (Møller and Mousseau, 2013) have been recorded in areas around Chernobyl. In Fukushima impacted coastal areas, a decline in the abundance and density of sessile intertidal biota was recorded (Horiguchi et al., 2016). However, difficulties in assessing the combined impact of the tsunami and elevated radiation dose rates prevent a definitive understanding of the cause of the decline. The lack of a scientific consensus regarding the long-term environmental and human health impacts of nuclear accidents have caused heightened concern amongst the wider public regarding radiation safety (Drottz-Sjöberg and Sjoberg, 1990; Orita et al., 2015). It is imperative that radioecology studies reporting no effect are regarded in the same manner as those finding detrimental radiation effects. This will enable a balanced judgement of the risk posed by anthropogenic radiation in the environment.

Reproductive endpoints are commonly used in environmental and toxicological studies owing to the potential for long term population level effects and ecological relevance (Lewis and Watson, 2012). A number of studies have documented reproductive effects on biota at Chernobyl. Møller et al. (2005) studied reproduction of barn swallows, *Hirunda rustica*, at Chernobyl and found reduced clutch sizes, lower hatching success and a smaller brood size of hatchlings in Chernobyl population as compared to controls. In murine rodents, a number of studies reported perturbations to reproduction and elevated embryonic mortality (Krylova et al., 1991; Testov and Taskaev, 1990), though effects appeared to be limited to the initial phase after the accident. In aquatic systems, gonadal abnormalities including asymmetry and oocyte resorption were positively correlated with contamination levels in a range of fish species including roach, *Rutilus rutilus*, perch, *Perca fluviatilis* and the goldfish *Carassius auratus* over a period from 1992 to 2005 (Belova et al., 2007). Tsytsugina and Polikarpov (2003) studied modes of reproduction and cytogenetic effects on populations of three species of Oligochaeta in contaminated areas in 1995–1996. The authors described a shift from asexual to sexual modes of reproduction at contaminated sites in two species; reproductive output was not directly quantified. This study, however, sampled only a single contaminated and control site precluding a robust understanding of the drivers of observed reproductive effects. To the authors' knowledge, no study has directly studied the impact of chronic radiation exposure on the reproduction of aquatic invertebrates at Chernobyl.

Members of the subphylum Crustacea are abundant in aquatic ecosystems globally and are gaining prominence as model organisms owing to increasing knowledge of crustacean genomics and biological systems. The International Commission on Radiological Protection (ICRP) developed the concept of reference animals and plants (RAPs) to use model organisms as a systematic basis for developing environmental radioprotection measures (ICRP, 2008). Due to their ubiquity in aquatic environments and well characterized biology, a marine crustacean of the family Cancridae has been selected as one of eight RAPs, highlighting the importance of understanding radiation effects on crustaceans for both members of the scientific and regulatory communities. At present, the effects of environmentally relevant doses of radiation on crustaceans are poorly understood (See Fuller et al., 2015 for review) owing to a lack of long-term studies in contaminated environments. In a previous study, no evidence of developmental effects (as measured using fluctuating asymmetry) of radiation exposure on the isopod crustacean, *Asellus aquaticus* at Chernobyl (Fuller et al., 2017) were found. The present study aimed to assess the effects of chronic radiation exposure on the reproduction of *A. aquaticus*. *A. aquaticus* is a benthic detritivore widespread in freshwater systems across Europe, playing a fundamental role in leaf litter degradation and nutrient cycling (Graça et al., 1993). *A. aquaticus* are widely considered to be semelparous (Chambers, 1977; Murphy and Learner, 1982; Steel, 1961 but see Maltby, 1991), with both the duration of both the breeding period and duration of embryo development driven by temperature (Andersson, 1969; Murphy and Learner, 1982; Økland, 1978). *A. aquaticus* has been used as a model organism in ecotoxicology in response to a range of toxicants including polycyclic aromatic hydrocarbons (De Lange et al., 2005), pesticides (Lukančič et al., 2010) and heavy metals (Van Ginneken et al., 2015).

Previous studies of *A. aquaticus* inhabiting polluted environments have recorded effects on reproduction. Populations below a coal mine effluent displayed a lower reproductive effort (defined as mg/offspring per mg/female) and modification of life history toward fewer larger offspring, which appeared to have a genetic basis (Maltby, 1991). Similarly, Tolba and Holdich (1981) recorded a lower fecundity in *A. aquaticus* individuals collected from sites with higher degrees of pollution. Controlled laboratory exposures have demonstrated a range of deleterious effects of chronic alpha and gamma radiation on crustaceans including delayed reproduction, reduced fecundity and survival of offspring (Alonzo et al., 2006, 2008; Gilbin et al., 2008 and Parisot et al., 2015). To the author's knowledge, no study has empirically tested the effects of radiation on crustacean reproduction following chronic low-dose radiation exposure in the field. Based on laboratory studies, we hypothesised that *A. aquaticus* individuals exposed to radiation at Chernobyl for thirty generations would display reduced reproductive output and altered timing of reproduction. To test this hypothesis, *A. aquaticus* individuals were collected from six lakes along a gradient of radionuclide contamination in Belarus and the Ukraine and fecundity, brood mass and maternal weight were assessed. Maternal weight was used as a proxy of reproductive timing (Donker et al., 1993).

2. Materials & methods

2.1. Sampling sites & collection of *Asellus aquaticus*

A. aquaticus was collected from six lakes along a gradient of radionuclide contamination in Belarus and the Ukraine in May – June of 2015 and 2016 (See Supplementary Information, Fig. S1). Lakes were selected based on long-term exposure to a gradient of radiation doses and historical measurements of radioactivity. Sites were visited once per sampling year and samples collected by kick netting in littoral zones from three different sub-sites at each lake using a 1 mm mesh size net (EFE, UK). The number of gravid (individuals bearing eggs) and non-gravid females collected is displayed in Table S1. The same sub-sites were visited the following year. Individuals were sorted lakeside and immediately preserved in 96% ethanol in individual Eppendorf tubes. A full description of the sampling sites is available in Fuller et al. (2017).

2.2. Hydrochemical parameters

A range of different hydrochemical variables including conductivity, oxygen saturation, pH and temperature were performed in situ using a multiparameter probe (HANNA Instruments 9828; see Table 1) at three sub-sites of each lake.

2.3. Calculation of radiation dose rates

A full description of the methods used to determine external and internal dose rates to *A. aquaticus* at sampling sites are provided in Fuller et al. (2017). Briefly, calculations were made using decay corrected deposition values of radiocaesium and strontium and dose conversion coefficients (DCCs) derived from geometry data of *A. aquaticus* using the ERICA tool (v 1.2, See Supplementary Information Table S2, Fig. S2). Internal dose rates were calculated based on average, decay corrected measurements of ^{137}Cs and ^{90}Sr in lakes at various depths taken during previous sampling in 2003. Total dose rates (individual absorbed dose rate) are provided in Table 1.

2.4. Reproductive output in *Asellus aquaticus* at Chernobyl

Adult (>3 mm in length, Hasu et al., 2007) *A. aquaticus* individuals were first sexed following Bertin et al. (2002) by analysis of the pleopods. Individuals were measured and weighed using the Leica Application Suite (v 4.5) and a Kern ABT 120-5DM (DE) analytical fine balance with a precision of ±0.02 mg respectively. Embryos were removed from the marsupium using a glass Pasteur pipette and photographed individually using a Leica DFC310 camera. Individuals were then reweighed to provide an estimate of the total weight of the brood. Embryos were staged following the method of Holdich (1968). A total of 354 gravid individuals were analysed over the two years of sampling (See Supplementary Information, Table S1).

2.5. Statistical analyses

Differences in brood sizes and weights between sites were tested using linear mixed effects models via the nlme package (Pinheiro et al., 2015) in R Studio Version 1.01 (R Studio Team, 2016). Sampling site, year and developmental stage of the brood were used as fixed effects and maternal weight as a random effect. In crustaceans, larger females typically produce greater numbers of eggs (e.g. Oh and Hartnoll, 1999). Owing to heterogeneity in the relationship between maternal weight and egg parameters between lakes in the present study, a random slopes and intercepts model was used. Models were fit using maximum likelihood methods and validated by analysis of residuals at each level of the random effect. Post-hoc multiple comparisons were used with Tukey contrasts via the multcomp package (Hothorn et al., 2008).

Differences in the percentage of females with broods between sites of varying contamination and sampling years was tested using binary logistic regression with the glm function. Female reproductive status (gravid = 1, non-gravid = 0) was used as the binary dependent variable where site and sampling year were predictors. Differences in the body mass of gravid *A. aquaticus* between sample sites were tested using a Kruskal-Wallis and post-hoc Dunn's test with Benjamini-Hochberg correction via the dunn.test package (Dinno, 2017).

Where significant differences between sites of varying contamination were present, the relationship between measured environmental characters and reproductive parameters were tested using linear regression. This was conducted on pooled environmental data for all sub-sites. Where assumptions of regression were violated, spearman's rho was used. All statistics were conducted in R Studio (R Core Development Team, 2016).

3. Results

3.1. Proportion of breeding females

The proportion of females with broods was not related to radiation dose rate (Fig. 1, linear regression F = 3.262, df = 1, 10, $r^2 = 0.246$ $p = 0.101$). The number of adult *A. aquaticus* with broods was significantly different between sites (logistic regression, $\chi = 8.65$, df = 5, $p < 0.001$), but not between sampling years ($\chi = 1.85$, df = 1, $p = 0.065$). The most contaminated site, Glubokoye, had the greatest proportion of females with broods over two years of sampling (Mean ± SEM 56.4 ± 13.9%), significantly greater than all sites excluding Svatoye 3 (49.1 ± 4.7%, Post-hoc Tukey's contrast, $p > 0.05$). The lowest proportion of females with broods was recorded at Stoyecheye (10.3 ± 7.3%). The proportion of females with broods was not correlated with any of the measured environmental parameters; dissolved oxygen (F = 0.3447, df = 1, 10, $r^2 = 0.033$, $p = 0.570$), water temperature (F = 0.357, df = 1, 10, $r^2 = 0.034$, $p = 0.564$), conductivity (F = 0.925, df = 1, 10, $r^2 = 0.085$, $p = 0.359$) and pH (F = 0.107, df = 1, 10, $r^2 = 0.011$, $p = 0.750$).

Table 1
Radiation dose rates (individual absorbed dose rate) and environmental parameters at six lakes along a gradient of radionuclide contamination at Chernobyl. Values for environmental parameters are presented as mean ± SD for values taken at three sub-sites of each lake.

Site	Sampling date	Dose rate (μGy/h)	Temperature (°C)	Oxygen saturation (%)	pH	Conductivity (μS/cm)
Gorova	11/6/2016	0.064	22.4 ± 0.05	113 ± 16.2	8.60 ± 0.02	256 ± 0.41
	23/6/2015	0.064	22.2 ± 0.05	185 ± 26.5	9.69 ± 0.02	179 ± 0.30
Dvorische	29/05/2016	0.691	23.2 ± 0.06	80 ± 1.17	7.60 ± 0.17	197 ± 0.15
	11/06/2015	0.786	23.7 ± 0.06	68.9 ± 1.00	7.82 ± 0.17	200 ± 0.00
Stoyecheye	27/05/2016	0.774	22 ± 0.05	102 ± 2.00	8.30 ± 0.02	241 ± 1.48
	08/06/2015	0.872	24.1 ± 0.06	89.4 ± 1.76	8.70 ± 0.02	230 ± 1.41
Svatoye 3	24/05/2016	2.03	20.1 ± 0.23	92 ± 1.80	8.00 ± 0.15	114 ± 0.70
	05/06/2015	2.2	23 ± 0.70	81.6 ± 1.60	7.8 ± 0.14	122 ± 0.75
Yanovsky Crawl	05/06/2016	20.42	20.2 ± 0.11	140 ± 2.90	9.00 ± 0.04	265 ± 0.97
	19/06/2015	20.6	23.3 ± 0.11	109 ± 2.25	9.40 ± 0.04	275 ± 1.00
Glubokoye	03/06/2016	26.4	23.6 ± 0.06	112 ± 14.10	7.60 ± 0.18	199 ± 1.22
	16/06/2015	27.1	24.9 ± 0.06	66.3 ± 8.32	7.92 ± 0.19	185 ± 1.14

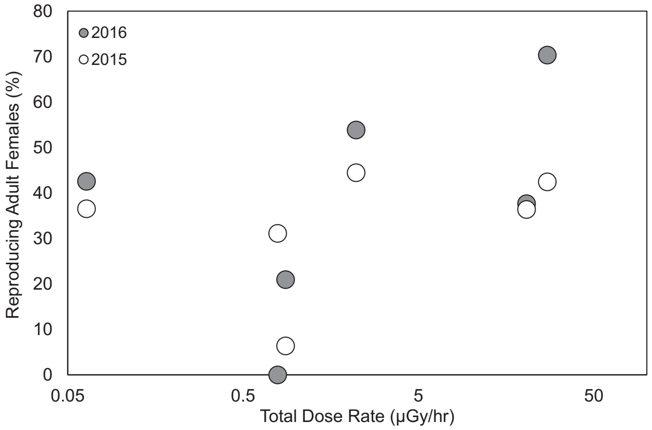

Fig. 1. Scatterplot of the relationship between the proportion of reproducing female *Asellus aquaticus* and total dose rate at six sites of varying contamination at Chernobyl.

3.2. Fecundity

The number of eggs (brood size) produced by female *Asellus aquaticus* did not vary between sampling year ($F_{1,80} = 7.5$, $p = 0.740$, See Supplementary Information Tables S3, S4) or with the developmental stage of the brood ($F_{1,\,80} = 0.602$, $p = 0.502$). The greatest number of eggs was produced at Glubokoye Lake over the two sampling years (Mean ± SD, 6.477 ± 2.259 eggs per mg maternal weight, See Fig. 2) with the fewest eggs being produced at Stoyecheye Lake (4.811 ± 1.824 eggs per mg maternal weight), however no significant effect of sampling site on brood sizes was recorded ($F_{1,\,80} = 2.402$, $p = 0.494$). This was further emphasised by the lack of relationship between total dose rate and brood sizes (Spearman's rank-order correlation, rho = 0.008, $p = 0.877$).

3.3. Brood mass

Brood mass did not vary significantly between sampling years ($F_{1,\,70} = 3.653$, $p = 0.441$) or between sampling sites of varying radionuclide contamination ($F_{1,\,70} = 0.562$, $p = 0.456$, See Fig. 3). Developmental stage was found to have a significant effect on the mass of the brood however ($F_{1,\,70} = 25.060$, $p = 0.0018$). This was owing to a significantly (Post-hoc Tukey contrasts, $p = 0.0018$) greater mass of broods in the final stage of development (Mean ± SE, 2.847 ± 0.590 mg) compared to the first stage of development (1.045 ± 0.073 mg). This effect was independent of sampling site as indicated by the non-significant interaction between sampling site and developmental stage ($F_{1,\,70} = 0.642$, $p = 0.426$).

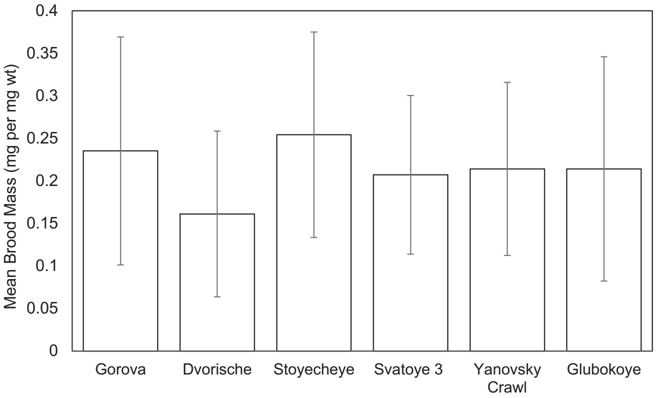

Fig. 3. Mean brood mass normalised to maternal weight of *A. aquaticus* collected from six sites along a gradient of radionuclide contamination. Sites are plotted in order of increasing contamination from left to right. Error bars represent standard deviations.

3.4. Maternal body mass

No significant differences in the body mass of gravid *Asellus aquaticus* were recorded between sampling years (Kruskal-Wallis test, $\chi^2 = 0.347$, df = 1, $p = 0.558$). Significant differences in maternal body mass were recorded between sampling sites (Kruskal-Wallis test, $\chi^2 = 109.4$, df = 5, $p = 0.000$), owing to a significantly greater mass of *A. aquaticus* at Stoyecheye (18.938 ± 6.904) and Svatoye 3 (11.730 ± 4.735) compared to all other sites (See Fig. 4, Dunn's test, p = 0.000). Differences in body mass were not related to total radiation dose rate (Spearman's rank-order correlation, rho = −0.081, $p = 0.129$) or any of the other measured environmental variables; conductivity (rho = −0.062, $p = 0.245$), temperature (rho = −0.077, $p = 0.149$), pH (rho = 0.108, $p = 0.449$) and dissolved oxygen (rho = 0.878, $p = 0.878$).

4. Discussion

The research hypothesis for this work was that populations of the isopod crustacean *Asellus aquaticus* chronically exposed to ionising radiation at Chernobyl would display reduced reproductive output and altered timing of reproduction. In order to test this hypothesis, gravid females were collected from six sites along a gradient of radionuclide contamination in areas impacted by Chernobyl in 2015 and 2016. Reproductive output was assessed and weight of gravid females was

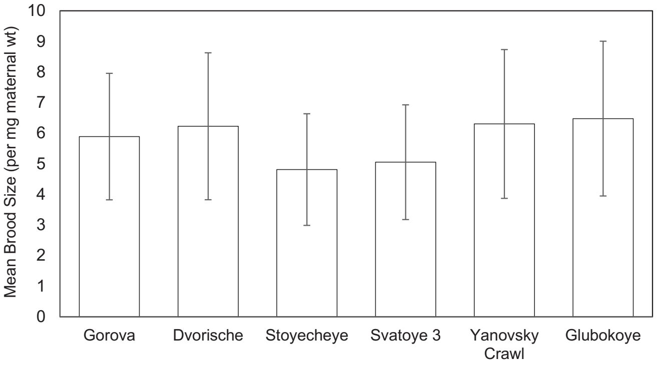

Fig. 2. Mean brood sizes normalised to maternal weight in *Asellus aquaticus* from six sites along a gradient of radionuclide contamination in Belarus and the Ukraine. Sites are plotted in order of increasing contamination from left to right. Error bars represent standard deviation.

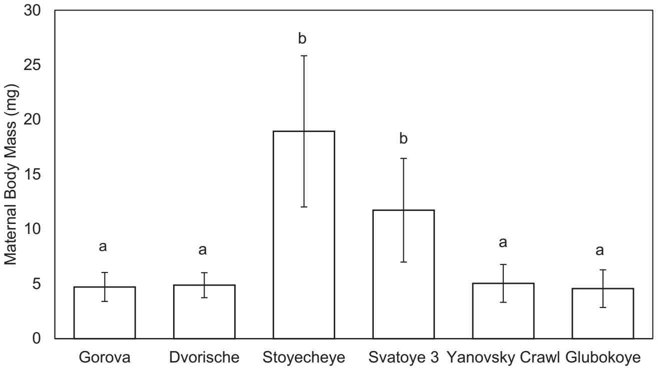

Fig. 4. Body mass of gravid *A. aquaticus* from six sites along a gradient of radionuclide contamination in Belarus and the Ukraine. Matching letters represent no significant difference, different letters represent significant differences (Dunn's test, $p < 0.05$). Error bars are ±SD.

used as a proxy of timing of reproduction. No significant differences in the numbers or mass of offspring produced at sites of varying radionuclide contamination was found in the present study. Significant differences in the body mass of ovigerous females was found, suggesting different timing of reproduction between localities. However, this was not related to radiation dose rates or any of the measured potential confounding factors. No support for these hypotheses were found within the data.

The present study finds no evidence that current dose rates in aquatic systems at Chernobyl (maximum total dose rate of 27.1 μGy/h, See Table 1) are impacting the reproduction of *A. aquaticus*. However, reproduction of *A. aquaticus* populations may have been affected immediately following the accident. Dose rates from sediment in aquatic systems in the immediate aftermath of the accident were estimated to be in the range of 100–200 mGy/d. Based on laboratory studies of crustaceans exposed to radiation (e.g. Parisot et al., 2015), such dose rates would be anticipated to cause deleterious effects on reproduction, though drastic differences in sensitivities between crustacean species have been recorded (See Fuller et al., 2015 for review).

A range of different organisations have proposed dose thresholds and benchmarks below which no detrimental impacts on populations of aquatic organisms are expected. For example, UNSCEAR concluded that maximum dose rates of ≤400 μGy/h to an individual within an aquatic population would not have detrimental effects at the population level, owing to a lack of evidence suggesting significant effects on reproduction at ≤200 μGy/h (Copplestone et al., 2008; UNSCEAR, 2008). For the ICRP's reference crustacean (ICRP, 2008), a derived consideration reference level (DCRL) of 400–4000 μGy/h has been proposed, within which there is some chance of deleterious effects to individuals. The lack of observed effects in the present study at dose rates of up to 27.1 μGy/h is consistent with these benchmark values. Lower dose benchmarks of 10 μGy/h have been proposed as generic screening values for environmental radioprotection in the absence of detailed organism-specific data (ERICA & PROTECT projects, Larsson, 2008; Howard et al., 2010). In this study of reproductive output in a single crustacean species, no negative effects at dose rates higher than those generic values were observed. Though the possibility of adaptation of populations at Chernobyl cannot be ruled out, this suggests that these lower benchmarks may be overly conservative for protection at the population level for some groups of organisms. Emphasis should therefore be placed on the development of species specific benchmarks for protection of the environment.

Recent studies have documented reproductive effects in crustaceans chronically exposed to radiation doses below those in the present study. Parisot et al. (2015) demonstrated reduced fecundity and delayed reproduction in *Daphnia magna* following multigenerational laboratory exposure to ^{137}Cs at 7 μGy/h. Two of the six sampling sites in the present study exceeded this dose rate (Yanovsky Crawl and Glubokoye Lake, see Table 1) with no detectable reproductive effects. Disparities between controlled laboratory studies and those conducted in the field have been reviewed by Garnier-Laplace et al. (2013). In contrast to the present study, the authors found greater sensitivity to radiation in organisms studied in the field compared to those exposed under controlled conditions. One possible explanation for the differences between the two studies may be adaptation of *A. aquaticus* to chronic radiation exposure. *A. aquaticus* populations are univoltine, meaning approximately thirty generations have occurred since the nuclear accident (Brattey, 1986). Crustaceans have been shown to gain tolerance to pollutants in as few as seven generations (Sun et al., 2014), further suggesting adaptation as a mechanism for the lack of effect in the present study. This highlights the necessity of field studies to validate and contextualise the results of laboratory experiments. Further comparative research into the effects of low-dose radiation on crustacean reproduction in the lab and field will enable a greater understanding of the importance of adaptation in species response to radiation.

In the present study, no significant relationship between measures of female reproduction and a range of environmental parameters was recorded. In *A. aquaticus,* duration of both the breeding period and embryonic development is related to temperature and the number of degree days above a minimum temperature (Andersson, 1969; Murphy and Learner, 1982; Økland, 1978). Studies have further suggested a role of photoperiod and food availability in governing reproductive patterns in *A. aquaticus* (Tadini and Valentino, 1969). In the present study, sampling was conducted once per year at each locality over two sampling years. Analysis of additional environmental conditions throughout the year such as food availability and the timing of spring would allow for greater understanding of the factors driving *A. aquaticus* reproduction within these lakes, and may explain the lack of a relationship between environmental parameters and *Asellus* reproduction. However, sampling throughout the year was not possible owing to logistical and permitting restrictions. In the majority of reported cases *A. aquaticus* individuals reproduce only once during their lifespan and are considered semelparous (Chambers, 1977; Murphy and Learner, 1982; Steel, 1961) though multiple broods have been recorded (e.g. Maltby, 1991). Therefore, sampling once per year is adequate to gain an understanding of typical reproductive output in *A. aquaticus.*

The lack of effect on reproduction in the present study may suggest that metabolic resources are being diverted from other processes in order to meet the physiological costs of radiation exposure (Jones and Hopkin, 1996). Similarly, alterations to metabolic requirements may lead to changes in feeding behaviour. Nascimento and Bradshaw (2016) demonstrated reduced grazing activity in *Daphnia magna* exposed to acute gamma radiation from ^{137}Cs. Alonzo et al. (2006) further suggested a metabolic cost of alpha radiation exposure in *D. magna.* Given the importance of feeding in *A. aquaticus* on leaf litter decomposition and therefore nutrient cycling in freshwater ecosystems (Graça et al., 1993), future studies should focus on the metabolic impacts of radiation exposure at Chernobyl and knock-on effects on feeding in *A. aquaticus*. The advent of advanced techniques such as metabolomics in environmental toxicology (e.g. Cappello et al., 2013; Xu et al., 2015) enables greater understanding of the metabolic pathways affected by stressors in the environment. Given the previous studies suggesting a metabolic cost of radiation exposure, future studies should employ a metabolomic approach to assessing the effects of radiation on biota.

5. Conclusions

In conclusion, the present study did not observe any significant association of reproductive endpoints in crustaceans with radiation. This suggests either that there are no such effects in current *Asellus* populations, or that these effects are so subtle to be undetectable in the natural environment given other environmental influences on *Asellus* reproduction. This study is the first to monitor reproduction in crustaceans at Chernobyl. The results of this study will aid in understanding the long-term effects of radiation exposure at the population level and support the management and monitoring of radioactively contaminated environments.

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Conflict of interest statement

This project was funded by the UK Natural Environment Research Council, Radioactive Waste Management Ltd. and the Environment

Agency of England and Wales. J.T.S. has also carried out small (<£10 k) consultancy projects for a range of clients including the Japan Atomic Energy Agency and UK Radioactive Waste Management Ltd.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.309.

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FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Postgraduate Research Student Handbook for more information

Postgraduate Research Student (PGRS) Information		**Student ID:**	615590
PGRS Name:	Neil Fuller		
Department:	Biology	**First Supervisor:**	Professor Alex Ford
Start Date: (or progression date for Prof Doc students)		**01/10/2014**	
Study Mode and Route:	Part-time ☐ Full-time ☒	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐

Title of Thesis:	The Biological Effects of Ionising Radiation on Crustaceans: Combining Lab and Field Studies
Thesis Word Count: (excluding ancillary data)	42,868

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:

Ethical review of project has been conducted by NERC. Also submitted to University ethics committee, pending confirmation

Signed *(PGRS)*:		**Date:** 23/05/2018