Effects of chronic radiation exposure on *Daphnia*: from individuals to populations

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Summary abstract

Increasing human activity means that populations are more susceptible to sudden environmental change. However, we have an inadequate understanding of the long-term effects of human-induced change on natural populations. This thesis focuses on the long-term effects of exposure to high environmental radiation levels resulting from the Chernobyl disaster. The accident caused both abrupt environmental changes to populations from high acute exposure from the initial radionuclide release and long-term chronic exposure from radionuclides that persist in contemporary populations. This work tests radiation effects by combining laboratory and field approaches using *Daphnia*, a freshwater crustacean, as a model organism.

I first examined the fitness of Chernobyl *Daphnia* populations sampled from eight lakes with different radiation levels and found that variation across populations was not due to dose rate. Assessment of population genetics showed that genetic diversity increased with dose rate, indicative of mutation accumulation. However, gene flow between populations reduced population structure, which could explain why no phenotypic differences were observed between populations. I then tested radiation effects under controlled laboratory conditions. This required the characterisation of the irradiation facility at Stirling University. Testing under continuous radiation exposure revealed a reduction in *Daphnia* survival across generations, consistent with mutation accumulation. Assessment of reproductive fitness revealed that inferior lineages were selectively removed from the experiment, stripping variation in reproductive effects.

This thesis demonstrates that ionising radiation negatively impacts individual *Daphnia* lineages in the laboratory at dose rates relevant to highly contaminated areas in the Chernobyl Exclusion Zone (350 µGy h^{-1} in the laboratory, <~180 µGy h^{-1} estimated field dose rate to *Daphnia*), and also found that genetic diversity was higher in wild populations experiencing higher dose rates. However, my field research also uncovered evidence that is consistent with the idea that the negative radiation effects are masked by selection from other ecological pressures.

Declaration of authorship

I, Jessica Goodman, declare that this thesis has been composed by myself and that it embodies the results of my own research. Where appropriate, I have acknowledged the nature and extent of the work carried out in collaboration with others

Signed…….

Date ……

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Chapter one:

General Introduction

1.1 Protection of the environment from ionising radiation

Radiation is energy that that can travel through space in the form of particles or waves (Hussein, 2007; Kudryashov, Yurii, 2008). Radiation with enough energy to cause damage at the molecular level is referred to as ionising radiation (NRC, 2006). Some level of ionising radiation is always expected to be naturally present in the environment and this level is termed background radiation. Both natural radiation sources such as cosmic rays and radon gas, and anthropogenic sources including radiation released from medical procedures and from the production of nuclear power, contribute to total radiation dose (Eisenbud & Gesell, 1997; UNSCEAR, 1982).

The International Commission on Radiological Protection (ICRP) is an organisation that provides recommendations on protection from ionising radiation. Radiation protection standards were primarily focused on human protection (ICRP, 1977), under the stance that "if humans are adequately protected from ionising radiation, then other species are unlikely to be put at risk" (ICRP, 1991). However, in 2008, ICRP recommended that radiation protection should be extended to wildlife (ICRP, 2008), presenting new challenges in assessing radiation effects across different species.

In order to begin addressing these challenges, ICRP created a framework for protecting different species. The framework was designed to be compatible with the existing radiation protection standards for humans. This framework is based on concept Reference Animals and Plants (RAPs) (ICRP, 2008). The RAPs are a set of organisms selected to represent an appropriate generalisation of different species. The 12 RAPs include wild grass, pine tree, bee, earthworm, deer, rat, duck, frog, trout, brown seaweed, crab and flatfish. Taxonomically, *Daphnia* are listed as crustacea and this demonstrates the limitations of using these 12 RAPs. However, the data presented in this thesis will contribute to the general understanding of radiation effects and can be used to test the robustness of the RAPs approach.

Different exposure pathways (such as inhalation through air, transfer through skin in aquatic environments or external exposure through emersion into water, NDAWG, 2009) and radiosensitivities were considered, to designate points of reference ("benchmarks"). Benchmarks are used to contextualise the levels of radiation risk and are usually numeric values given as doses or dose rates (see Box 1). Benchmarks are usually either legal benchmarks, where the designated levels should not be exceeded (there are currently no limits in place for the protection of wildlife) or used as screening values. Screening values are generally conservative estimates, where negligible radiation effects are expected below these values. Values exceeding screening value

benchmarks will not necessarily have detrimental effects but more research may be required to understand the level of risk (Garnier-Laplace *et al.*, 2010; ICRP, 2014).

1.2 Units of ionising radiation

The International System of units is most widely used to measure different aspects of ionising radiation. The two key parameters measured are activity and exposure. The activity of a radioactive source is measured in Becquerel (Bq), where one Bq is equal to one disintegration per second. Units of radiation exposure are expressed in different ways, depending on radiation effects and variation in sensitivity. For wildlife, only the absorbed dose is used. The radiation dose to an organism (absorbed dose) is a measure of radiation exposure expressed in Gray (Gy), where one Gy is equal to the absorption of one joule of radiation energy per kilogram of matter (BIPM, 2006; NRI, 1999; Greening, 1985; ICRU, 1998).

Radiation effects can vary depending on the type of radiation administered and the tissue type that has been affected. To address issues in estimating radiation effects, ICRP generated values for radiation weight factors for the radiation type (W_R) to calculate an Equivalent dose and the affected tissue (W_T) to calculate the Effective dose for humans (ICRP, 1977, 2007). Equivalent and Effective doses refer to radiation exposure that is specific to humans, given in Sieverts (Sv). These doses cannot be physically measured but can be calculated from Gy. Equivalent doses are calculated by multiplying the absorbed dose by W_R. Effective dose is calculated by multiplying the Equivalent dose by W_T (Henriksen & Maillie, 2003). Dose conversion coefficients are used to calculate equivalent values for non-human biota, based upon the RAPs proposed by ICRP (see section 1.1) (ICRP, 2017)

Non SI units (used in older literature and in American publications) used units for measuring ionising radiation include the Curie (Ci), radiation absorbed dose (rad), Roentgen (R) and the rem. Ci is a unit for the activity of a source (similar to Bq), where one Ci is equal to 37,000 MBq (Rutherford, 1910). Rad units are used for radiation exposure in terms of absorbed energy (similar to Gy), where one Gy is equal to 100 rad (ICRP, 1955, 1964). The Roentgen (R) is also a unit of radiation exposure, however it refers to the ionisation of air molecules and therefore cannot be used as a dose measurement (as in Gy or rad, Henriksen & Maillie, 2003). Rem was introduced in 1962 by ICRP as an equivalent dose for rad, which was later changed to the Sv (ICRP, 1977).

1.3 Dosimetry

There are a variety of different methods that can be used to detect ionising radiation levels. More commonly used methods for environmental radiation detection can be categorised as either integrating dosimeters or pulse mode detectors. Alternative dosimetry methods are generally more suited to other applications, for example, ionisation chambers are commonly used in radiotherapy for calibration purposes (Karger *et al.*, 2010). An example of an integrating dosimeter used in Chapter four is a thermoluminescent dosimeter (TLD). TLD's are frequently used as personal dosimeters due to their high sensitivity and wide range of detectable doses (Kortov, 2007). Crystals contained within TLD's trap released electrons when they are exposed to ionising radiation. Heating these crystals enables them to release electrons which then emit light (Meijvogel, van der Burg and Bos, 1996; Bos, 2001). Thermoluminescence is a useful tool for measuring ionising radiation because the light emitted is proportional to the amount of radiation that is absorbed. Lithium fluoride (LiF) was the first substance to be used in TLD's due to similarities in absorption to soft tissue. Other commonly used materials now also include Aluminium oxide (Al_2O_3) and Calcium fluoride (CaF_2) (Kortov, 2007).

Pulse mode detectors include scintillators, Geiger Müller counters and semiconductor detectors (reviewed in Knoll, 1979). Geiger Müller and scintillator counters are used in Chapter two, to detect levels of ionising radiation in Chernobyl in the air and in water samples respectively. Geiger Müller counters consist of a gas-filled cylinder that conducts electricity when it comes into contact with a high energy particle (Geiger and Muller, 1928). They are useful in detecting environmental radiation levels but do not provide information on the type of radiation present. Scintillation methods work on the principle that different substances will emit light after exposure to ionising radiation. A sensitive light detector (usually a photomultiplier tube) converts the light into an electrical signal which can then be measured. Scintillation counters can identify gamma isotopes because the light emitted is proportional to the amount of gamma radiation absorbed (Ageno *et al*., 1950; Reynolds *et al*., 1950).

It is also possible to model radiation dose through computer simulations, which are often coupled with dose measurements for verification. One of the most common approaches for the mathematical modelling of dose rate from ionising radiation sources is the Monte Carlo modelling technique, specifically Monte Carlo Neutron Transport (MCNP) models (Briesmeister, 1986; Hendricks *et al.*, 2000). This process is utilised in Chapter four, where TLD measurements are used to verify predictions made using the

MCNP approach. The process simulates the exposure pathways of ionising radiation (from protons, neutrons, photons, electrons, etc.) emitted from the source, including scattering and absorption interactions with the materials making up the sample and its surroundings. Each interaction is then used to make a dose calculation. MCNP modelling of ionising radiation used in a wide range of applications, including radiotherapy, shielding design and assessment and nuclear facility assessments (Rogers *et al.*, 1995; da Silva and Crispim, 2001; Ancius *et al.*, 2005; Gilbert *et al.*, 2012; Tekin and Manici, 2017). MCNP has consistently been proven as an accurate method for predicting radiation dose in a range of scenarios (Whalen, Hollowell and Hendricks, 1991; Nathan *et al.*, 2003; Kiger *et al.*, 2005; Chetty *et al.*, 2007; Szoke *et al.*, 2014).

1.4 Genetic effects of ionising radiation

Ionising radiation can cause a variety of DNA lesions, such as DNA-protein crosslinks, damaged base sites, single and double strand breaks (Nikjoo *et al.*, 2001; von Sonntag, 2007), with the DNA double strand break being the most biologically damaging (Olive, 1998). Once DNA damage has been caused, DNA damage response pathways are activated which may include cell cycle arrest and apoptosis (Iliakis *et al.*, 2003). More commonly, genes involved in DNA damage repair will be activated and attempt to repair the lesion. Ionising radiation can cause mutation through damaging the DNA directly, through pitfalls in DNA repair machinery causing alterations to the DNA sequence or chromosomal translocations (Hakem, 2008) and through indirect mechanisms such as reactive oxygen species (ROS, DOE *et al*., 2000; Feinendegen, 2002).

In addition to directly damaging the DNA, internal repair mechanisms can also contribute to the damage caused by ionising radiation. Although some mutations can occur in non-coding regions of the DNA or may not alter the amino acid sequence produced due to the triplicate nature of the genetic code, mutations that alter the DNA sequence within coding regions of the DNA that result in a change in product, may result in damaging effects. Repair machinery usually maintains DNA integrity by using the complementary DNA strand to reconstruct the damaged sequence (Alberts, 2003). There are a number of mechanisms that maintain DNA integrity following damage from radiation exposure. Single strand breaks can be repaired through a number of mechanisms including direct reversal of DNA damage (Mishina *et al*. 2006), base excision repair (Seeberg *et al*. 1995), DNA mismatch repair (Kunkel & Erie 2005) and nucleotide excision repair (Costa 2003). Double strand breaks are repaired through

either homologous recombination or non-homologous end-joining mechanisms. Homologous recombination has a higher accuracy, whereas non-homologous end joining is more efficient and takes less time (Mao *et al.*, 2008). Both these types of repair mechanisms are conserved across a wide range of species, although in higher eukaryotes, non-homologous end-joining is more important for double strand break repair than homologous recombination (Shrivastav, De Haro and Nickoloff, 2008; Iliakis, 2009). *Daphnia* utilise both mechanisms for DNA damage repair (Nakanishi *et al.*, 2014, 2015).

Homologous recombination works by re-joining the DNA ends at the break site and restoring the sequence in between (Shinohara and Ogawa, 1995). The essential components for homologous recombination are searching for homology between two sequences, invading the strands by the Rad51-single-stranded DNA presynaptic filament and strand invasion at the 3' end of the template DNA. RAD54 promotes DNA synthesis at the end of the process (Sung and Klein, 2006; Li and Heyer, 2008). Homologous recombination restores the original sequence and prevents chromosomal translocations. However the utilisation of sister chromatids during homologous recombination restricts the process to the S and G2 phases of the cell-cycle, whereby DNA is replicated and distributed between two daughter cells during mitosis (Mao, *et al*., 2014).

During the process of non-homologous end-joining, the broken ends of DNA are brought together and ligated without the requirement of a homologous template strand to repair a double strand break. Therefore, non-homologous end-joining operates at any phase of the cell cycle. It is also a shorter process than homologous recombination and even some single-strand break repair processes (Hefferin and Tomkinson, 2005). Despite the apparent advantages of non-homologous end-joining over homologous recombination, the enzyme involvement in the process means that if two double strand breaks occur within a close proximity, it is possible that the DNA ends may not join up correctly, resulting in chromosomal translocation (Mao, *et al*., 2008; Mao *et al*., 2014) Additionally, DNA damaging agents such as ionising radiation often damage the nucleotide bases and in some cases, the phosphate backbone of the DNA which can result in nucleotide addition, loss or alteration. These damaged break sites therefore require additional processing before ligation can occur, potentially resulting in an overall sequence alteration.

Ionising radiation can indirectly induce DNA damage through the induction of ROS such as hydrogen peroxide, hydroxyl radical and superoxide (Riley, 1994; Dent *et al.*,

2003). ROS reacts with biomolecules including DNA, lipids and proteins, which can result in increased DNA damage and responses at the cellular level including senescence and apoptosis (Chen *et al.*, 1998; Chen *et al*., 2000; Mates and Sanchez-Jimenez, 2000). ROS also contributes to genomic instability (see section 1.5), which can be transmitted to future generations and may have detrimental effects on progeny (Morgan, 2003a; b). Transgenerational impacts of ionising radiation, with regards to the transmission of mutations to offspring is assessed in Chapter five.

1.5 Non-genetic effects of ionising radiation

Ionising radiation also induces non-genetic effects such as, epigenetic changes and bystander effects. Epigenetics describes heritable alterations to DNA expression which are not attributed to genetic change (Waddington, 1957). Epigenetic profiles will change throughout normal development and in response to environmental stress such as starvation or pollution (Lumey *et al.*, 2007; Heijmans *et al.*, 2008; Bind *et al.*, 2013; Suarez-Ulloa *et al*., 2015). Epigenetic modifications include DNA methylation (which suppresses gene expression, reviewed in: Bird, 1986), histone acetylation (which increases gene expression, reviewed in: Struhl, 1998) and gene silencing through long non-coding RNA's (reviewed in: Mercer *et al.*, 2009).

Although epigenetic changes are associated with ionising radiation (Dubrova *et al.*, 2000; Barber *et al.*, 2002; Barber and Dubrova, 2006), the mechanisms are largely unknown. Research suggests that epigenetic effects of ionising radiation in both animals and plants are predominantly non-adaptive (reviewed in: Youngson and Whitelaw, 2008). DNA methylation is one of the epigenetic processes that regulates gene expression, where DNA hypomethylation results in an upregulation of gene expression (Razin, 1998). Most research suggests that global DNA hypomethylation occurs following radiation exposure (Raiche *et al.*, 2004; Koturbash *et al.*, 2006), including *Daphnia* experiments (Trijau *et al.*, 2018). However, more detailed methylation studies have shown that hypo/hypermethylation is variable across different tissue types (Pogribny *et al.*, 2004; Zielske, 2015). In addition, epigenetic effects of low radiation doses have previously been extrapolated from high-dose exposures, whereas increasing evidence at low doses suggests that DNA methylation profiles neither increase or decrease with radiation dose, but differential methylation patterns arise across genes (Waldren, 2004; Ma *et al.*, 2010; Antwih *et al.*, 2013).

Bystander effects refer to the process where irradiated cells/organisms transfer signals to non-irradiated cells/organisms. Bystander responses include apoptosis, mutation, genomic instability and chromosomal rearrangements (Morgan, 2003a, 2003b; Morgan

and Sowa, 2007). The mechanism for such effects still largely remains unclear, although ROS have been implicated in the process (Narayanan *et al.*, 1997; Azzam *et al.*, 2003).

1.6 Ionising radiation effects at the organism level

Ionising radiation effects at the molecular level may result in radiation effects at higher levels of biological organisation (*i.e.* individual, population, ecosystem). Typical assessments at the level of organism are conducted on growth, reproduction and survival, similar to standard ecotoxicology assessments (Garnier-Laplace *et al.*, 2008). These traits are highly representative of the overall fitness of an organism and have been used for assessing organism fitness throughout this thesis. Research generally shows that ionising radiation has detrimental impacts on these measures of fitness (*e.g.* Phillips & Coggle, 1988; Zaka *et al.*, 2004; Parisot *et al.*, 2015), consistent with research at the sub-organism level (sections 1.4 and 1.5).

Furthermore, studies at the level of organism have revealed “subtle” radiation impacts such as reproductive delays, age at maturity and embryo development (Taskaev *et al.*, 1988; Florian. Parisot *et al.*, 2015; Hurem *et al.*, 2017). These life stage specific alterations suggest that life history evolution might be influenced by the effects of ionising radiation. Whilst there has been no radiation-specific mechanism related to such effects identified to date, there is abundant evidence that oxidative stress impacts life-history evolution. Specifically, relating to the balance between investment in somatic maintenance and survival (Alonso-Alvarez *et al*., 2004, 2006; Dowling & Simmons, 2009; Monaghan *et al*., 2009). Evolutionary theory also tells us that organisms investing in somatic maintenance can show reduced reproductive investment (Williams, 1957; Kirkwood, 1977; Harman, 1981; Kirkwood and Rose, 1991). However, whilst this provides potential explanation for more nuanced effects on organism fitness, evidence for radiation-mediated life history shifts in wild populations is limited (*e.g.* Blaylock, 1969; Cooley, 1973).

1.7 Ionising radiation impacts on evolutionary processes

Organism and sub-organism level assessments are useful because data can be extrapolated to the population level (Birch, 1948; Beckerman *et al.*, 2002; Alonzo, Hertel-Aas, *et al.*, 2008). However, it is important to consider that organisms living within high radiation environments may be exposed to ionising radiation across many generations, potentially at variable levels of exposure (due to radionuclide decay and movement across a heterogeneous landscape). Exposure over generations may add further complications, as evolutionary processes such as selection and adaptation can

have effects at the population level. Ionising radiation causes mutations which supply variation to populations, so higher genetic diversity might be expected in populations experiencing high levels of exposure (Haldane, 1937; Kimura and Maruyama, 1966). However, exposure to radiation across multiple generations might also drive selection towards more radiation-tolerant genotypes and thus lower genetic diversity (Haldane, 1937; Crow, 1970).

One of the key challenges associated with assessing evolutionary impacts of ionising radiation is the effects of confounding factors in wild populations, that also drive evolutionary change such as parasitism, predation and competition (Brockelman, 1975; Lehmann, 1993; Creel and Christianson, 2008; Auld *et al.*, 2013), which may also impact overall population fitness. Policies for environmental radiation protection are created based on protecting populations from radiation risk rather than individuals (Beresford *et al.*, 2007; Garnier-Laplace *et al.*, 2008). Therefore, it is important that radiation impacts on populations are conducted accurately, considering all potential confounding factors. One way of addressing this issue is the use of genetic techniques on populations living across a variety of radiation contamination levels. By assessing parameters such as genetic diversity and population structure in relation to dose rate, it should be possible to test whether radiation is a key factor in shaping population fitness. This approach is used in Chapter three, to assess population diversity in Chernobyl populations of *Daphnia*.

1.8 Controversy in current approaches for radiation assessment on wildlife

Whilst confounding evolutionary processes have previously been considered in radiation studies in natural environments (Polikarpov, 1998), there are still issues that need to be addressed in terms of assessing radiation in the context of the wider ecosystem (Mothersill *et al.*, 2019). Particularly because there has been controversy surrounding some of the reported wildlife effects (Beresford and Copplestone, 2011). Møller and Mousseau frequently report effects on wildlife at dose rates considerably lower than expected (*e.g.* Møller & Mousseau, 2007, 2009; Moller & Mousseau, 2011). This issue was highlighted in a review of laboratory and field data from radiation studies, where data from research conducted by Møller and Mousseau's group are clear outliers on the dose response curve (Garnier-Laplace *et al.*, 2013).

Møller and Mousseau have received several criticisms that could explain the differences between their reported dose rates at which effects are observed, and those of the rest of the radioecology community. One suggestion is that these differences could be attributed to the dosimetry approaches used, specifically that only external

measurements of dose rate were taken into account (Beresford *et al.*, 2008; Beresford and Copplestone, 2011; Garnier-Laplace *et al.*, 2013). Additionally, work conducted on bird abundances at Fukushima (Møller *et al.*, 2012) has been criticised for the interpretation of statistical analysis. Specifically, that the low R^2 value does not support the significant results presented and that high degrees of freedom suggest that confounding variables have not been taken into account (Beresford *et al.*, 2012). Møller and Mousseau received further criticism on their work which showed increased cataract frequency with ionising radiation in voles (Lehmann *et al.*, 2016). This was also due to statistical interpretations, as a significant relationship was presented despite the lack of effects in male voles and a weak relationship in females. There were also few animals tested from control sites and the nature of the preservation methods used have been suggested to compromise lens opacity and hence results interpretation (Smith *et al*., 2016). These suggestions might explain why the dose rates reported by Møller and Mousseau are lower than those reported by other researchers working in the Chernobyl Exclusion Zone (CEZ, Wickliffe & Baker, 2011).

It is important to take confounding factors into account. Additional stressors that are naturally present can act synergistically or antagonistically with ionising radiation, either exacerbating or masking radiation impacts on populations (Holmstrup *et al*. 2010; Coors and De Meester 2008). Radiation studies should consider a multi-stressor approach to understand some of these impacts, in line with current ecotoxicology research (Mothersill *et al.*, 2019). Additionally, common garden experiments (also known as transplant experiments) offer a useful solution to issues involving confounding factors in wild populations (de Villemereuil *et al.*, 2016). These experiments involve testing organisms in a common environment. The purpose of these experiments is usually to test local adaptation strategies (de Villemereuil *et al.*, 2016), but they can also be used to assess organism fitness in the absence of confounding factors (as demonstrated in Chapter two).

In natural environments the distribution of contamination is likely to be heterogeneous due to variation in radionuclide transport in different environmental conditions (Mccarthy and Zachara, 1989; Thiessen *et al.*, 1999). The movement of organisms across their natural landscapes should be considered in the dosimetry, for example, terrestrial organisms will be exposed to different levels of radiation as they move across a heterogenous landscape of contamination. This could lead to further issues in reporting effects. *Daphnia* are used as a model species throughout this thesis, as populations can be defined by the boundaries of the water body they inhabit, with more

restricted migration than in terrestrial species. This may reduce some of the variability in dose rate predictions.

1.9 Daphnia as a model organism

Daphnia are a useful model organism for studying radiation effects and offer a variety of benefits to both laboratory and field-based studies that address some of the issues in predicting radiation effects on populations. *Daphnia* live in freshwater lakes and ponds on every continent (Adamowicz *et al.*, 2009). They are a key species for studying aquatic ecosystems and play a central role in food-web dynamics (Miner, de Meester, *et al.*, 2012). Effects on *Daphnia* life history traits therefore have the potential to cause significant responses in the surrounding environment (Pace *et al.*, 2004; Flaherty and Dodson, 2005).

Daphnia reproduce on a cyclically parthogenetic basis, meaning that they have both sexual and asexual components of their reproductive cycle, triggered by environmental cues (Hebert, 1987, see Figure 1.1). Throughout the spring/summer *Daphnia* populations will reproduce asexually where their offspring are genetically identical to each other and to the parent. It is possible to maintain field-sampled *Daphnia* asexually in the laboratory (*i.e.* the same genotype that exists in the field) for testing in the absence of confounding factors. *Daphnia* will begin their sexual cycle as conditions become less favourable (*i.e.* during autumn/winter); they will produce male offspring which will mate with the females to produce sexual eggs (Berg, 1931). Sexual eggs are encased within hard capsules termed ephippia, which each contain two eggs that get released into the water body and remain dormant throughout the winter (Alekseev and Lampert, 2001; Decaestecker *et al*., 2009). Ephippia can withstand extremely hostile conditions including freezing and desiccation (Meijering, 2003; Altermatt and Ebert, 2008). Migration of *Daphnia* between populations is restricted to the resting stages (ephippium) of *Daphnia* reproduction, where eggs be carried through means such as wind or by predator species such as birds (Maguire, 1963).

Different substances or conditions can induce “sub-lethal” alterations to life history aspects such as growth, reproduction and survival which can be easily monitored as a proxy for fitness of individual *Daphnia* (Flaherty and Dodson, 2005; Beasley *et al.*, 2015). The asexual component of their reproductive cycle provides the opportunity to generate natural experimental replicates that can be exposed to a variety of different treatment conditions, making them a valuable tool in the laboratory. *Daphnia* can therefore be used in a wide variety of toxicity studies including; the effects of metals and metal accumulation (Liu and Wang, 2015; Okamoto *et al*., 2015), as well as the

toxicity of pharmaceuticals and organic pollutants within water systems (Flaherty and Dodson, 2005; Pérez *et al*., 2015). *Daphnia* reproduction is also used as an OECD test for chemical toxicity (OECD, 2012), demonstrating their application as a useful organism for assessing benchmark values.

Daphnia pulex also have a fully annotated genome sequence, allowing researchers to investigate environmental effects on gene function (Colbourne *et al.*, 2011). A variety of molecular applications exist for *Daphnia*, creating the opportunity to investigate differences in population genetics across landscapes (Shaw *et al.*, 2008). This is useful in uncovering any evolutionary processes that could mask variation in phenotype. There have also been advances in the methylation profile of *Daphnia pulex*, showing changes in response to the environment. This highlights applications of *Daphnia* as an epigenetic model (Asselman *et al.*, 2015; Strepetkaitė *et al.*, 2015). The benefits of asexual reproduction make it possible to investigate changes to the epigenome without the complication of confounding genetic factors (Harris *et al*., 2011).

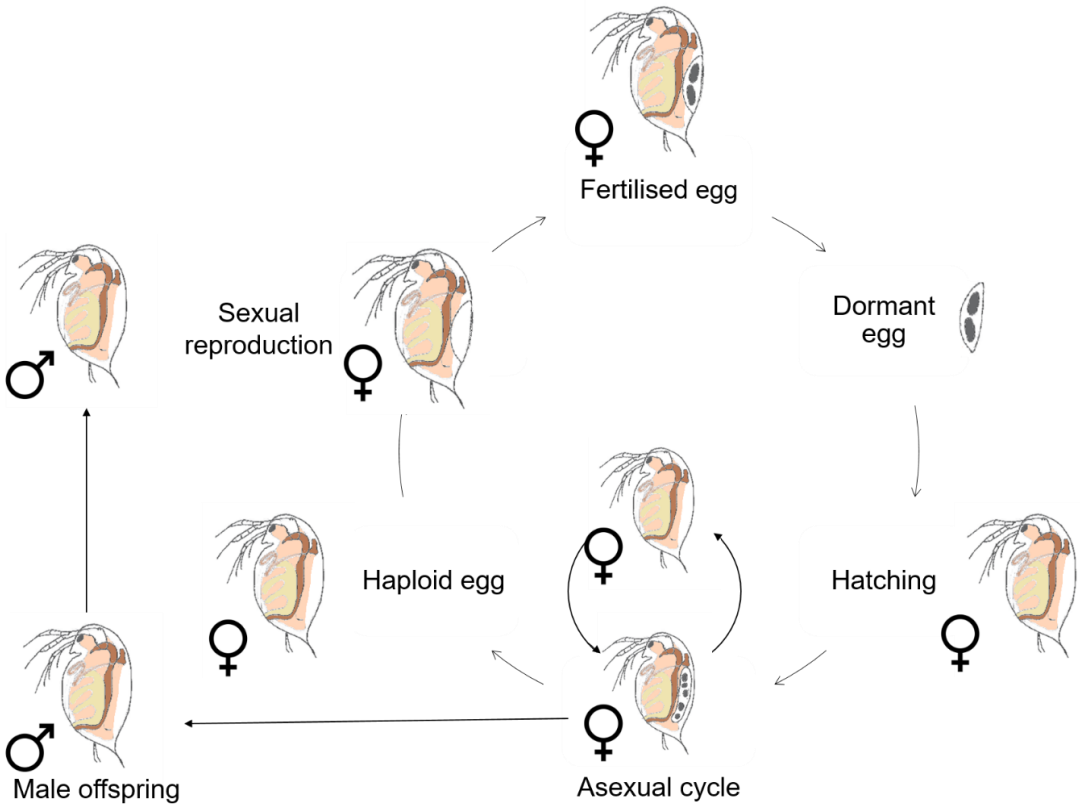

Figure 1.1: *Daphnia* lifecycle. Adapted from Vizoso (2005).

1.10 Ionising radiation in the context of other anthropogenic and natural stressors

The field of radioecology can benefit from the existing research in ecotoxicology based on the use of multiple stressor approaches and assessing evolutionary responses to abrupt environmental change. Ecosystems are usually exposed to multiple stressors including both natural stressors (such as predation, food availability and parasitism: Lehmann, 1993; Oro *et al.*, 2003; Creel & Christianson, 2008; Auld *et al.*, 2013) and anthropogenic stressors (such as heavy metals and chemical pollutants, Nazir *et al.*, 2015; Noyes & Lema, 2015). The requirement for a multi-stressor approach is widely recognised in ecotoxicology (Eggen *et al.*, 2004; Holmstrup *et al.*, 2010; Baird *et al.*, 2016; Van den Brink *et al.*, 2016), whilst it has only recently been highlighted as a key issue for the assessment of radiation effects (Bréchignac, 2016; Mothersill *et al.*, 2019). One of the main challenges in implementing this approach is the controversy in assessment of radiation effects in wild populations (see section 1.8), as correct dose response curves would need to be established to model radiation with other stressors (Garnier-Laplace *et al.*, 2013; Goussen *et al.*, 2016).

Ecotoxicology research can also provide insight into the evolutionary responses to long-term radiation exposure in the environment. This is partly because some anthropogenic stressors have similar effects on wildlife to ionising radiation. For example, air pollution, heavy metal and pesticides have all been shown to generate mutations (Kada, Moriya and Shirasu, 1974; Shirasu *et al.*, 1976; Lin *et al.*, 1994; Yang *et al.*, 1999; Somers *et al.*, 2002; Gómez-Martín *et al.*, 2014) and reactive oxygen species (Risom, Møller and Loft, 2005; Valko, Morris and Cronin, 2005; Liu, Zhu and Wang, 2015; Mangum *et al.*, 2015) in wildlife. In addition, relevant assessments on the effects of chemical pollutants, including mutagenic substances, on population genetic structure provide a useful insight into evolutionary responses to long-term exposures (Giska *et al.*, 2015; Inostroza *et al.*, 2016). Furthermore, literature on population responses to abrupt environmental change is valuable in predicting the effects of unscheduled radiation releases (such as accidental releases) on wildlife (Husseneder *et al*., 2016; Reid *et al.*, 2016).

It is also important to consider that the research being conducted on radioactively contaminated sites following nuclear accidents, including the work described throughout this thesis, is valuable in providing information to other fields of research on responses to abrupt environmental change. The CEZ provides a unique opportunity to understand both the immediate population responses to rapid changes in the

environment (Kozubov *et al.*, 1987; Shevchenko *et al.*, 1992; Arkhipov *et al.*, 1994), as well as long term population effects (IAEA, 2006a; Møller & Mousseau, 2006; Beresford & Copplestone, 2011; Omar-Nazir *et al.*, 2018). This thesis provides insight into the long term phenotypic (Chapter two) and genetic (Chapter three) responses to ionising radiation in wild populations and tests radiation effects in isolation (Chapters four and five), to characterise radiation effects alone. This could inform other researchers on long term responses to sudden high concentrations of pollutants, for example following an oil spill, metal contamination or industrial acidification (Esler *et al.*, 2002; Pollard, Colbourne and Keller, 2003; Riffaut *et al.*, 2005; Keller *et al.*, 2007).

1.11 Chernobyl as a natural laboratory

The Chernobyl disaster occurred on the 26th April 1986. During an experiment to test how the electricity system would cope in the event of an emergency, a combination of faults led to the explosion of reactor four (Smith & Beresford, 1989; Warner & Harrison, 1993). As a result of the Chernobyl disaster, approximately 1.85x10^{18} Bq of different radionuclides were released into the atmosphere at the time of the accident (IAEA, 2006a). This resulted in high levels of contamination in the surrounding areas of Belarus, Russia and Ukraine and radionuclides were deposited more widely across Europe, after being transported by the weather and deposited through precipitation (Wheeler, 1988; ApSimon *et al.*, 1989).

Initial exposures from the accident were acute, dominated by short-lived radioisotopes such as ^{140}Ba, ^{133}Xe and ^{131}I, and resulted in negative impacts on biota (Hinton *et al.*, 2007; Geras'kin *et al*., 2008). There were detrimental effects, ranging from increased mutation rate to high incidences of mortality across arrange of species following the initial phases of radiation exposure (Kozubov *et al.*, 1987; Krivolutsky *et al.*, 1990; Testov & Taskaev, 1990). An early concern for humans following the accident was ^{131}I, which directly affects the thyroid gland through ingestion and inhalation pathways. Despite preventative efforts to avoid the uptake of iodine through contaminated dairy products and produce in the surrounding areas of Chernobyl, increased screening showed a significant increase in thyroid cancer in children (Warner & Harrison 1993; Nikiforov & Gnepp 1994; Pacini *et al*. 1997; Jacob *et al*. 1998).

Overall radiation levels rapidly declined following the first year of the accident due to radionuclide decay of the short-lived radionuclides. The remaining radionuclides were transported through the environment by processes such as precipitation leading to migration into deeper soil layers. Approximately one year following the Chernobyl disaster, the chronic exposure phase began, dominated by long-lived radionuclides

such as ^{137}Cs and ^{90}Sr (UNSCEAR, 1996). The heterogenous radionuclide deposition across the CEZ makes it a useful site for studying radiation effects across a contamination gradient (Appendix A).

Generally, studies in the literature have used dose rates measured on site when reporting effects on wildlife. However, it is inappropriate to report that these dose rates are directly responsible for the tested effects (or lack thereof) because organisms in the CEZ are not naïve to radiation and have experienced a complex evolutionary exposure history. This is explored further in Chapter two. As outlined previously, evolutionary processes such as selection and adaptation will have had an influence on wild populations that are present in the CEZ now.

1.12 Objectives of the project

This project uses *Daphnia* as a model organism to test whether current chronic and historic radiation doses in the CEZ impact on individual and/or population fitness. Firstly, assessment of fitness levels of natural *Daphnia* populations living across a gradient of contamination is assessed in the CEZ (see Chapter two). Population genetic diversity is then assessed in Chapter three to determine whether any underlying molecular variation contributes to the results on phenotypic differences in fitness. Wild *Daphnia* populations have experienced radiation exposure across multiple generations. It is therefore important to capture this in the laboratory through transgenerational exposure to Chernobyl relevant dose rates (see dose terminology in Box 1). An irradiation facility containing a ^{137}Cs source was established at Stirling University in 2016. In order to conduct a transgenerational radiation study, the irradiation facility first needed to be characterised to determine doses for each treatment group and a dose response experiment (Chapter four) had to be conducted to determine appropriate dose rates for multiple generations of exposure. Results from the dose response study were used to inform the experimental design for transgenerational exposure of *Daphnia* to ionising radiation in Chapter five.

Box 1: Dose terminology

This thesis focuses on dose rates that could realistically occur in a natural environment. Classification of exposure levels are specific to this thesis and have been categorised in the context of wildlife living within the CEZ (see Chapters two and three).

Dose is the total amount of absorbed radiation (given in Gy throughout the thesis)

Dose rate is the absorbed radiation dose over a given time period (usually an hour and given in µGy h^{-1}).

Chronic exposures describe a duration of radiation exposure that lasts for a substantial part of an individual's lifespan.

Acute exposures usually refer to high dose radiation exposures over a short period. In this thesis, acute exposures refer to radiation exposure that lasts for a fraction of an individual's lifespan.

Levels of exposure according to dose rate:

Very low: < 1 µGy h^{-1}

Low: 1-10 µGy h^{-1}

Medium: 10 - 100 µGy h^{-1}

High: 100 - 200 µGy h^{-1}

Very high: 200 – 500 µGy h^{-1}

Chapter two:

Variation in chronic radiation exposure does not drive life history divergence among *Daphnia* populations across the Chernobyl Exclusion Zone

This chapter has published as:

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2.1 Abstract

Ionising radiation is a mutagen with known negative impacts on individual fitness. However, much less is known about how these individual fitness effects translate into population-level variation in natural environments that have experienced varying levels of radiation exposure. In this Chapter, I sampled genotypes of the freshwater crustacean, *Daphnia pulex*, from the eight inhabited lakes across the Chernobyl Exclusion Zone. Each lake has experienced very different levels of chronic radiation exposure since a nuclear power reactor exploded there over thirty years ago. The sampled *Daphnia* genotypes represent genetic snapshots of current populations and allowed me to examine fitness-related traits under controlled laboratory conditions at UK background dose rates. I found that whilst there was variation in survival and schedules of reproduction among populations, there was no compelling evidence that this was driven by variation in exposure to radiation. Previous studies have shown that controlled exposure to radiation at dose rates included in the range measured in the current Chapter reduce survival, or fecundity, or both. One limitation of this study is the lack of available sites at high dose rates, and future work could test life history variation in various organisms at other high radiation areas. My results are nevertheless consistent with the idea that other ecological factors, *e.g.,* competition, predation or parasitism, are likely to play a much bigger role in driving variation among populations than exposure to high radiation dose rates. These findings clearly demonstrate that it is important to examine the potential negative effects of radiation across wild populations that are subject to many and varied selection pressures as a result of complex ecological interactions.

2.2. Introduction

Populations are constantly challenged with selection from competitors, predators and parasites (Ball and Baker, 1996; McLaughlin *et al.*, 2002; Auld and Brand, 2017). An increase in human activities means that natural populations are also at a higher risk of sudden, dramatic changes to their environment (from events such as oil spills, chemical releases and climate change) (Bickham *et al.*, 2000; McLaughlin *et al.*, 2002; Riffaut *et al.*, 2005; Husseneder *et al.*, 2016), which can have detrimental impacts on individuals and thus populations (*e.g.,* Bickham & Smolen, 1994; Santos *et al.*, 2013).

Nuclear accidents such as those at Chernobyl and Fukushima are prime examples of human-induced dramatic environmental change. These accidents have resulted in widespread radioactive contamination of the surrounding areas. The levels of ionising radiation across these areas show considerable variation both over space, due to heterogeneity in radionuclide deposition and over time, as a result of radionuclide decay (Saxen *et al*, 1987; Saito *et al.*, 2015). Whilst negative effects of radiation on individuals are known (Breimer, 1988; Morgan, 2003a; b; von Sonntag, 2007), it is difficult to extrapolate effects on individuals to the level of the population (Bréchignac, 2016; Spurgeon, 2018). These difficulties arise because of two key issues: first, organisms living within high radiation environments (> 420 μGy h^{-1} Hinton *et al*., 2007) could exhibit a lower overall mean fitness due to physiological stress (Kimura and Maruyama, 1966) Second, strong selection for radiation-tolerant individuals could reduce differences in mean fitness between high- and low-radiation populations (Esnault *et al*., 2010; Galván *et al.*, 2014) and thus mask the negative effects of radiation on individuals. Indeed, strong selection for radiation-tolerant phenotypes may explain how some natural populations can persist in high radiation environments (Baker *et al.*, 1996; Murphy *et al*., 2011)

Ionising radiation also generates mutations, which are the founding source of all genetic variation (Haldane, 1937; Kimura and Maruyama, 1966). Variation in fitness-related traits in contemporary populations may therefore be exacerbated by exposure to radiation in the Chernobyl Exclusion Zone (CEZ). However, ionising radiation can also exert selection on populations, and the evolution of radiation tolerance may drive depletion in population genetic variation. Both the mean and variance in fitness-related traits can give valuable insight into the balance between mutation (which causes increased variance and lower mean fitness) (Kimura and Maruyama, 1966) and selection (reduced variance with either no difference or increased mean fitness) (Haldane, 1937; Crow, 1970). It is, however, important to note that whilst mutation is

the ultimate source of all genetic variation, radiation is just one of many possible agents of selection. Ecological factors such as parasitism, predation and competition are known to have impacts on population fitness and may outweigh any effects of radiation in wild populations (Brockelman, 1975; Lehmann, 1993; Creel and Christianson, 2008; Auld *et al.*, 2013). Moreover, these ecological factors can influence fitness indirectly *e.g.,* by selecting on the predators, parasites or prey of the focal organism rather than on the focal organism itself (Reznick *et al*., 1990; Ball and Baker, 1996). Still, by quantifying trait variation among organisms collected across a gradient of chronic radiation dose, the effects of radiation exposure on shaping fitness can be assessed at the population level.

The CEZ provides a useful natural laboratory to test how variation in ionising radiation shapes life histories and fitness across wild populations (section 1.11). The spatial heterogeneity in chronic radiation across the CEZ (Figure 2.1, Table 2.1) provides an opportunity to test for dose-dependent effects of ionising radiation on natural populations. There are, however, major challenges associated with testing the fitness impacts of radiation exposure using natural populations. For example, individuals frequently move across a patchy landscape of radiation, making it difficult to estimate the overall absorbed dose they experience (Hinton *et al*. 2007). I overcame this problem by studying *Daphnia pulex*, a freshwater crustacean that inhabits discrete ponds and lakes (with low inter-population migration; Haag *et al.*, 2006) where I could obtain reliable estimates of absorbed radiation dose. *D. pulex* provides other advantages (see section 1.9): it reproduces both sexually and asexually, where most reproduction is asexual, but sex is required to produce hardy resting eggs that can survive the winter (Zaffagnini, 1987). By collecting *Daphnia* from lakes and ponds across the Chernobyl area, I was able to obtain a genetic snapshot of populations that have experienced very different levels of chronic radiation (from < 0.1 to over 180 µGy h^{-1}) and conduct a common garden experiment where fitness related traits could be quantified under UK natural background radiation levels. Specifically, I measured survival and asexual reproduction over the course of the *Daphnia* lifespan. I then used these data to calculate the instantaneous rate of population increase, *r,* for each genotype (a useful proxy for overall fitness).

In this Chapter, I explore how *Daphnia* life-history traits reflect evolutionary responses to long-term radiation exposures across the CEZ, with particular focus on the opposing processes of selection versus mutational input. I tested whether selection played a primary role in shaping populations by examining whether the variation associated with population fitness (instantaneous growth rate, *r*) declines with dose rate. I also

examined whether radiation reduced mean population fitness by testing whether *Daphnia* fitness declines with dose rate, as would be consistent with previous studies that have demonstrated laboratory exposure to radiation reduces invertebrate fitness (Sarapultseva & Gorski, 2013; Nohara *et al.*, 2014; Parisot *et al.*, 2015).

2.3. Materials and methods

2.3.1 Study system

Daphnia are sensitive to environmental change and have thus proven an excellent model for ecotoxicology (Pace *et al.*, 2004; Flaherty and Dodson, 2005); indeed, *Daphnia* reproduction is used as an OECD guideline for testing the toxicity of various chemicals and pollutants (OECD, 2012). Furthermore, immigration of *Daphnia* between populations is generally limited to the diapausing stage of their reproductive lifecycle (Haag *et al.*, 2006), so individual *Daphnia* phenotypes are likely to have been shaped primarily by the immediate environment. Finally, *Daphnia* are cyclical parthenogens, whereby they reproduce asexually throughout the spring/summer and sexually to produce resting eggs which remain dormant over the Autumn/Winter (Alekseev and Lampert, 2001; Decaestecker *et al*., 2009). This mixed reproductive mode means one can take advantage of their asexual reproductive stage to take genetic snapshots of wild populations and then examine clonal lines in replicated common garden experiments under controlled conditions (*e.g.,* Auld *et al.,* 2013).

2.3.2 Field collections and radiation dosimetry

I collected 38 *Daphnia* genotypes from the eight inhabited lake populations that were identified as appropriate for *Daphnia* sampling in June 2016 and maintained them as isofemale lines (henceforth called lines, see Table 2.2 for information on genotypes per lake). Each of the eight populations have experienced different levels of chronic radiation exposure (see Figure 2.1, Table 2.1). *Daphnia* samples were collected at one-metre depths using a plankton net (net mesh: 0.25 mm, bag depth: 300 mm, outer frame: 250 mm diameter). The animals were transported to the laboratory in Chernobyl within three hours of sampling. Isofemale lines were then established by placing the *Daphnia* individually in 50 mL falcon tubes with water collected from the corresponding lake; these lines were allowed to propagate clonally. *Daphnia* lines were transferred to uncontaminated natural mineral water and fed *Chlorella vulgaris* algae for transport back to the laboratory at the University of Stirling (where the life history experiment took place). Once in Stirling, the *Daphnia* lines were maintained in a climate control facility under standard conditions without further exposure to radiation above UK natural background levels (20 °C on a 12:12 hour light: dark cycle in 80 mL of artificial

Daphnia media (ADaM, Klüttgen *et al.*, 1994). Highest recorded UK background dose rate was 0.18 µGy h^{-1} in 2017 (RIMNET, 2017). I replaced the media and fed each genotype with 5 mL of *Chlorella vulgaris* three times weekly. Each line was maintained under standard conditions for three generations to minimise phenotypic variation due to maternal effects.

To assess radionuclide concentrations at each sample site I extracted data, where available from the Ukraine atlas (Intelligence Systems GEO, 2008), for ^{137}Cs and ^{90}Sr (the dominant radionuclides in Chernobyl) and ^{241}Am and ^{239}Pu, which were considered representative of other radionuclides within the water column and upper sediment (IAEA, 2008). Where no data were available in the literature, sediment and water samples were taken at each sample site and transported to the Ukrainian HydroMeteorological Institute (UHMI) for analysis.

Water samples were analysed as follows. First, 5 – 25 L of water was collected at each sample site and passed through an on-line filtration system using a combination filter (Petryanov's FFP-15-1.5 prefilter + Blue Ribbon Grade paper filter) with a cartridge containing sorbent ANFEZH® to concentrate ^{137}Cs and ^{90}Sr. Following this, the cartridge was removed, and the filtered water was spiked with the radiochemical tracers ^{243}Am and ^{242}Pu and acidified to pH 2 with Nitric Acid followed by radiochemical separation. In the laboratory at the UHMI the filter and sorbent were dried at 105^{o} C to a constant weight, thoroughly mixed and packed in container for gamma-spectrometry analysis. Where radioactivity levels were high enough, a sub-sample of water was taken for direct gamma measurement.

Sediment samples were taken as sediment cores, using a Kayak type sediment corer (made at the UHMI) from the deepest lake location (verified by echo-sound measurements). Sediment core quality was assessed based upon two parameters; that there was no disturbance between the upper sediment along the core tube and that contrasting properties at the base of the core were present, indicating formation prior to the Chernobyl accident in 1986. In the UHMI laboratory, the sediment cores were sliced into sections (1-5 cm in size), freeze dried, homogenised and submitted for gamma spectrometry analysis. Representative subsamples from selected slices (0.5 - 1.0g) were taken for radiochemical analysis.

Radiometric analysis for ^{137}Cs and ^{241}Am was conducted using a gamma-spectrometer with HPGe detector GMX-40-LB (Ortec, USA). ^{90}Sr and transuranic elements (^{238,239,240}Pu and ^{241}Am) were preconcentrated using carbonate/hydroxides precipitation followed by serial extraction chromatography separation on Sr-Resin and TRU-Resin

(Eichrom, USA) with ^{90}Sr measured on a Liquid Scintillation Counter (TriCarb 2900TR, Perkin-Elmer, USA) according to established methods (Laptev *et al*., 2015) or alpha-spectrometry on Alpha-8 instrument (BSI, Latvia) after electrodeposition in the case of transuranic elements. Combined uncertainty of the ^{137}Cs, ^{90}Sr and transuranic element activity measurements did not exceed 10%, 20% and 30% respectively (activity concentrations are given in Table 2.3).

The dose rate from internal and external radionuclides was estimated using ERICA (version 1.2), a software program designed to estimate radiation risk to wildlife based upon a range of representative species (Beresford *et al.*, 2007; Brown *et al.*, 2008, 2016; ICRP, 2009). ERICA assessments were made by calculating dose rates based upon the activity concentrations provided and data on environmental radionuclide transfer. My calculations were based on the default reference organism, Zooplankton, within ERICA. Zooplankton was selected on the basis of the geometry and size of *Daphnia pulex* collected. Occupancy (which refers to the location of the organism within the lake) was changed to 75% surface sediment and 25% water column reflecting the fact the *Daphnia* population lies dormant throughout the autumn/winter as resting eggs in the surface sediment, before hatching in spring (Alekseev and Lampert, 2001), and that they vertically migrate throughout the water column (from sediment to water surface) to obtain food throughout the rest of the year (McLaren, 1963; Dawidowicz and Loose, 1992). These occupancy rates should have produced a conservative estimate of the dose rate as the majority of the radionuclides were expected to have accumulated within the lake sediment (Nazarov and Gudkov, 2009). Dose estimates are given in Table 2.1.

Table 2.1: The names of sampling sites, coordinates and estimated dose rates and radiation levels. Coordinates are given using the Universal Transverse Mercator (UTM) system using the WGS84 ellipsoid. Radiation level groupings are given for reference throughout the chapter.

Lake name	Co-ord N	Co-ord E	Total dose rate ($\mu Gy\ h^{-1}$)	Radiation level
Vediltsy	51.4352	30.83846	0.07 – 0.1	Very low
Yampol	51.2095	30.17667	0.20*	Very low
Glinka	51.2174	29.93713	1.17*	Low
Buryakovka	51.3978	29.8931	1.77*	Low
Semikhody	51.4151	30.0502	17.52 – 18.04	Medium
Krasnyansky	51.4429	30.07643	22.77 – 55.78	Medium
Azbuchin	51.408	30.11102	115.57 – 115.65	High
Gluboke	51.4454	30.06528	166.85 - 181.15	High

*This is based on available data where in some cases calculation of range was not possible

Table 2.2: Number of genotypes setup for each of the eight lake populations, number of non-reproducing individuals and the number of genotypes assessed in the experiment.

Lake population	Number of genotypes setup	Number of non-reproducing individuals	Proportion of non-reproducing individuals	Number of genotypes in the experiment
Buryakovka	5	11	0.275	4
Yampol	6	31	0.658	2
Vediltsy	4	10	0.25	4
Glinka	4	5	0.125	4
Semikhody	4	21	0.525	4
Krasnyansky	7	14	0.25	6
Azbuchin	5	14	0.35	4
Gluboke	3	10	0.417	2
Total	38			30

Table 2.3: ^{137}Cs, ^{90}Sr, ^{241}Am and ^{239}Pu activity concentrations in water and sediment samples collected from each lake site. Water (w) concentrations are in Bq l^{-1} and sediment (s) concentrations in Bq g^{-1} (dry weight).

Lake	^{137}Cs (w)	^{137}Cs (s)	^{90}Sr (w)	^{90}Sr (s)	^{241}Am (w)	^{241}Am (s)	^{239}Pu (w)	^{239}Pu (s)
Buryakovka	0.1	11	0.45			0.3	0	
Yampol	0.23	3.5	0.22			0.2	0	
Vediltsy	0.15	8	0.3			0.2	0	
Glinka	0.22	5	0.45			0.3	0	
Semikhody	0.5-1.0	90	6.5-7.5		2.80E-03	4	3.30E-03	
Krasnyansky	0.5-1.5	3700-7400	14-28	1480-3700		40-100		40-100
Azbuchin	3.3-3.6	7500-20000	80-500	4000-7500	8.00E-04	100-200	2.00E-03	100-200
Gluboke	2.0-6.5	550	90-110	200	20-80E-3	20	15-50E-3	

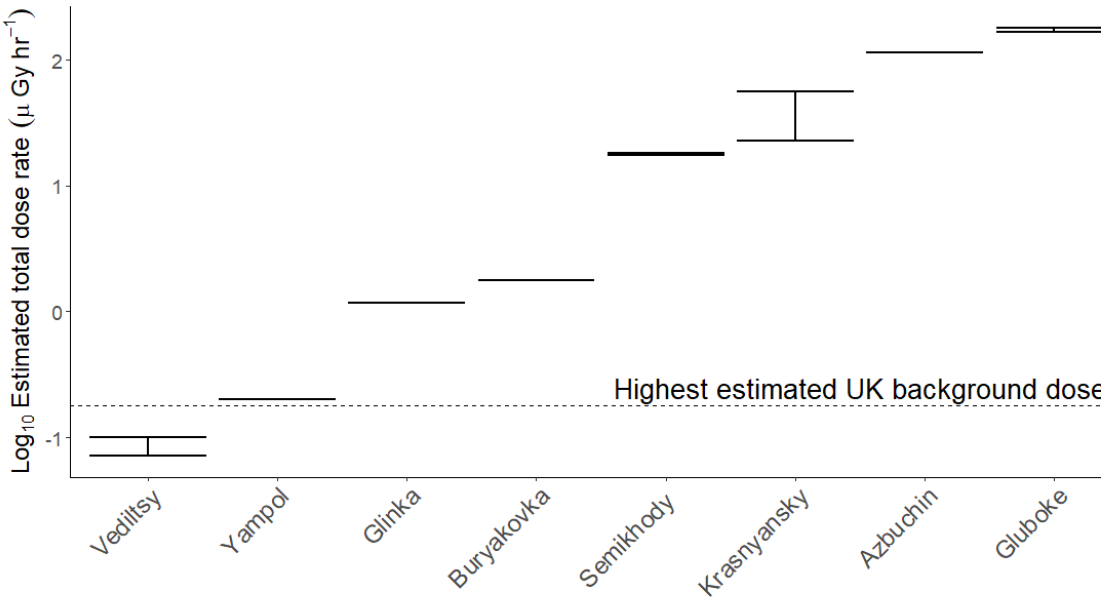


Figure 2.1: Log_{10} estimated total absorbed dose rates based on dose assessments made for each lake site, with ranges where appropriate (see Table 2.1). The black dotted line represents the highest estimated UK background dose rate of 0.18 µGy h^{-1} for comparison (Oatway *et al.*, 2010; RIMNET, 2017).

2.3.3 Life history experiment

On day one of the experiment, *Daphnia* neonates were assigned to fresh jars and maintained under standard conditions. Offspring from the third clutch from the third generation of *Daphnia* were used as experimental replicates to minimise variation due to maternal effects. Where maternal lines did not produce their third clutch from the third generation of *Daphnia* on day one of the experiment, the neonates were assigned to fresh jars thereafter, and the experimental days were standardised for statistical analysis. I measured the fecundity and survival of females daily from each of the 30 *Daphnia* isofemale lines from eight lake populations that had experienced different historical radiation doses. Fecundity was recorded as the day of each brood release and the number of offspring produced in each brood. Survival was measured by recording the day of death for each individual. There were eight replicates per line, where each replicate consisted of a single *Daphnia* in 50 mL of artificial *Daphnia* medium (ADaM; see Kluttgen *et al*., 1994). Replicate animals were fed 1.0 ABS *Chlorella vulgaris* algal cells per day (where ABS is the optical absorbance 650 nm white light) and the media was replaced when offspring clutches were released.

2.3.4 Statistical analysis

Analyses were performed using R statistical software (R Core Team, 2017) version 3.4.3. First, I tested the effects of dose rate and lake population on *Daphnia* survival. Specifically, I fitted mixed effects Cox's proportional Hazards (CoxME) models to the survival data (using the *coxme* package; Therneau, 2015, 2018), where dose rate was fitted as a covariate and lake population was fitted as a fixed effect. Line nested within lake population was included as a random effect to account for the fact that I measured multiple genotypes per lake. Significant effects of lake population were further investigated using a *post hoc* Tukey test to determine which populations were different from each other (using the *multcomp* package; Hothorn *et al.*, 2017).

The effects of dose rate and lake population on the total number of offspring produced were tested using generalised linear mixed models with Poisson error distribution (GLMM, implemented the *lme4* package; Bates *et al.*, 2015), where replicate within line within lake population was included as a random effect. Significant differences identified between lake populations were tested using a Tukey's range *post hoc* test. Using the same approach and random effects structure, but with a binomial distribution (as individuals were either identified as reproducing or not reproducing), I tested whether the number of non-reproducing individuals varied according to dose rate or lake population.

Next, I examined how dose rate and lake population affected age-specific reproduction using generalised additive mixed models (GAMMs within the *gamm4* package; Wood and Scheipl, 2017). GAMMs are semi-parametric models that are useful for predicting non-linear effects, where the linear predictor is dependent on a "smooth" function, which determines the level of smoothness in the fitted curve. This smooth function can depend on one or multiple non-parametric smoothers fitted to factors or covariates. I compared a model where smoothers were fitted to both experimental day and either dose rate or lake population to a model where a smoother was fitted to experimental day only. Random effects included replicate nested within line nested within lake population, to account for the fact that repeated fecundity measures were taken for each individual. In addition, I made pairwise comparisons of smoothed and unsmoothed models for combinations of pooled lake populations. The best fit model was determined using Akaike's information criterion (AIC), where the model with the lowest AIC was considered the best model and models with an AIC difference of less than two were regarded as the same (Burnham and Anderson, 2002).

Finally, I assessed overall population fitness by calculating the instantaneous rate of population increase (*r*) for each genotype using the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

Where x represents the age of each organism in days, l_x is the proportion of surviving females at each age classification and m_x is the number of offspring produced at each corresponding age (Birch, 1948; Grant and Grant, 1992; Cuco *et al.*, 2017). I tested for variation in *r* across lake populations and by dose rate using generalised least squares models (GLS models using the *nlme* package, Pinheiro *et al.*, 2018), where the intercept was allowed to vary by lake population. I tested for normality of distribution of *r* data using the Shapiro–Wilk and then performed a Bartlett's test to determine if variances in *r* differed according to lake population. Where dose rates were not normally distributed, a Fligner-Killeen test was performed to test if variance in *r* is associated with dose rate.

2.4 Results

2.4.1 Radiation exposure does not affect Daphnia survival

I found no effect of dose rate on *Daphnia* survival (CoxME: coefficient= -0.001 ± 0.004, z=0.15, p= 0.88). There were significant differences in survival across lake populations (CoxME: χ^2_7= 920.73, p < 0.0001, Figure 2.2. Median day of death in Vediltsy: 50, Yampol: 48, Glinka: 47, Buryakovka: 45, Semikhody: 59, Krasnyansky: 54, Azbuchin: 50, Gluboke: 45). Summary data for this chapter are included in Appendix B.

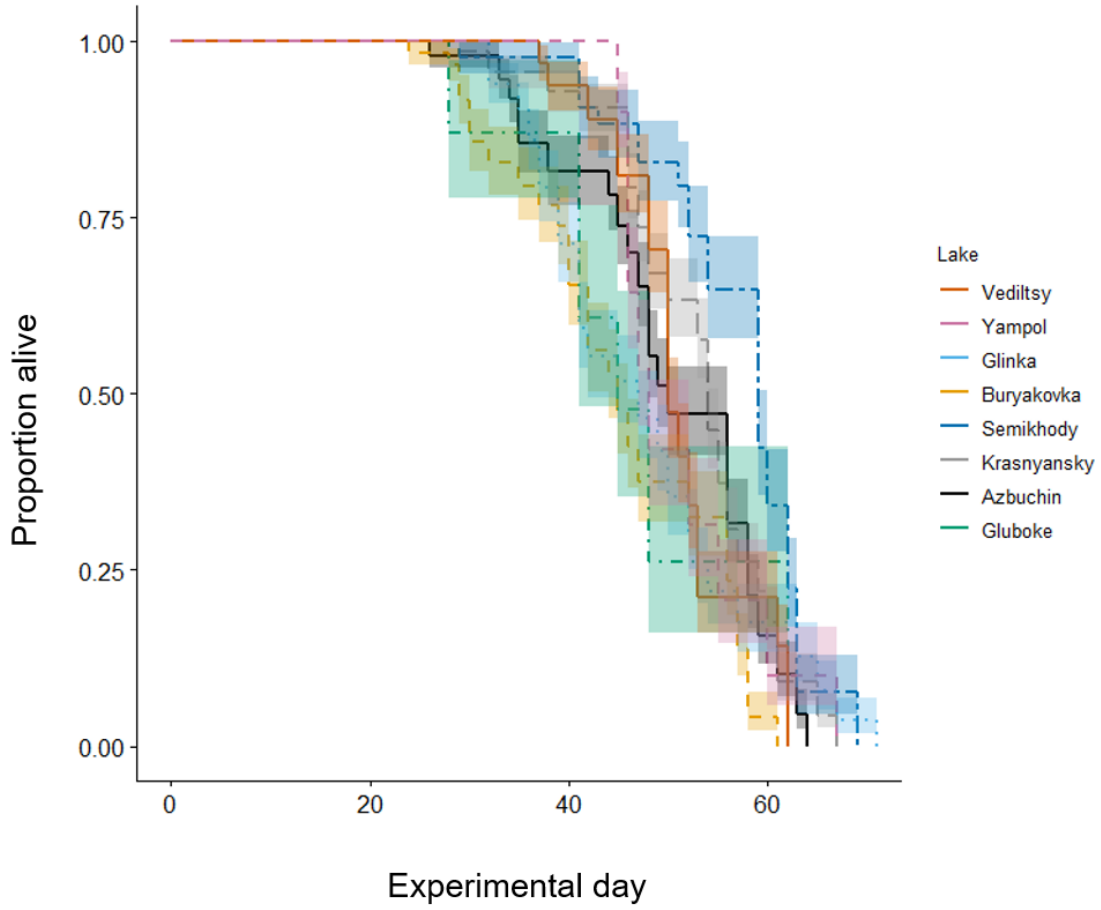


Figure 2.2: Effect of lake population on *Daphnia* survival (Survival probability; shaded regions denote ± 95% confidence intervals [CIs]).

2.4.2 Radiation exposure does not affect reproductive schedules

There was a significant effect of dose rate (GLMM: χ^2_1= 64.89, p < 0.0001) and lake population (GLMM: χ^2_7= 995.99, p < 0.0001) on the total number of offspring produced, though the variation in total offspring was better explained by population (ΔAIC= 981.99). Tukey's post-hoc test revealed that in all cases, this variation was driven entirely by lake Yampol (categorised as very low, p < 0.05 for comparisons between Yampol and all other lake populations. See Figure 2.3). The proportion of non-reproducing *Daphnia* varied between 0.125 and 0.658 across lines. Analysis found a marginally non-significant effect of dose rate on the likelihood of individual failure to reproduce (GLMM: χ^2_1= -3.6, p = 0.06); this suggests that if radiation-induced sterility does occur, it is unlikely to have a strong effect on population-level fecundity. By contrast, there were significant differences in the proportion of non-reproducing individuals among lakes (GLMM: χ^2_8= -31.67, p < 0.001). *Post hoc* testing revealed that

this was driven by a high incidence of non-reproducers in Yampol lake ($p < 0.05$. See Table 2.2).

Comparisons between models revealed that lake population explained more variation in age-specific reproduction than dose rate (see Table 2.4). Further, smoothing the day of reproduction by lake population significantly improved the model fit compared to fitting lake population as a parametric fixed effect (GAMM: $\Delta AIC = 482.18$, $\chi^2_{14} = 510.17$, $p < 0.0001$). The best fitting model included day by lake population as a non-parametric smoother and showed that all lakes varied from one another (Table 2.5), and that the timing of reproductive peaks varies across populations (Figure 2.4).

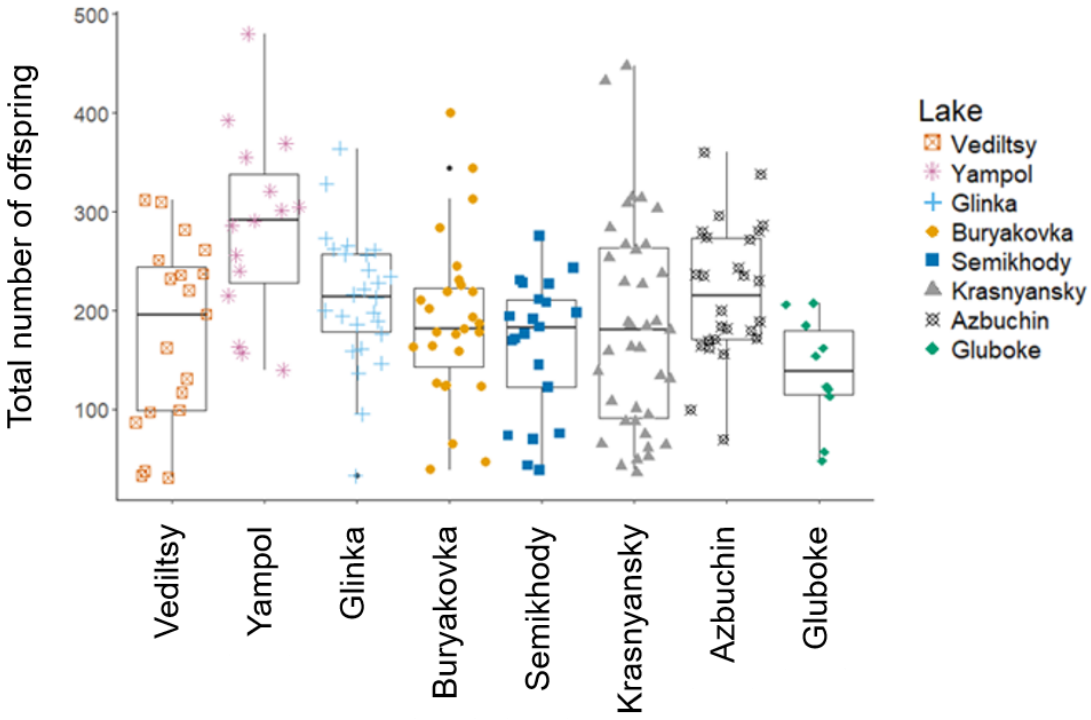


Figure 2.3: Boxplot showing the total number of offspring produced by each lake population. Populations are plotted in order of increasing dose rate. The box shows the upper and lower quartiles within the data and the line within each box shows the median value. The lines outside of each box show the range of the data.

Table 2.4: Summary for Generalised Additive Mixed Models (GAMMs) assessing age-specific reproduction. In all models, jar nested within line nested within lake is fitted as a random effect. N = 1899.

Response	Parametric/smoother	Term	AIC
Offspring production	Parametric	Dose rate	15635.83
	Smoother	Dose rate	17437.41
	Parametric	Lake population	15635.83
	Smoother	Lake population	15153.65

Table 2.5: Generalised Additive Mixed Model (GAMM) fitting age-specific reproduction data by lake population. Day by lake population is fitted as a non-parametric smoother and jar nested within line nested within lake is fitted as a random effect. eDF is the estimated degrees of freedom. N = 1899.

Response	Parametric/smoother	Term	df (eDF)	χ^2	p
Offspring production	Smoother	Day by Buryakovka	6.27	543.1	**<0.0001**
	Smoother	Day by Yampol	6.11	463.4	**<0.0001**
	Smoother	Day by Vediltsy	5.63	209.0	**<0.0001**
	Smoother	Day by Glinka	6.78	607.3	**<0.0001**
	Smoother	Day by Semikhody	7.28	221.6	**<0.0001**
	Smoother	Day by Krasnyansky	7.08	382.4	**<0.0001**
	Smoother	Day by Azbuchin	6.88	693.5	**<0.0001**
	Smoother	Day by Gluboke	5.46	127.6	**<0.0001**

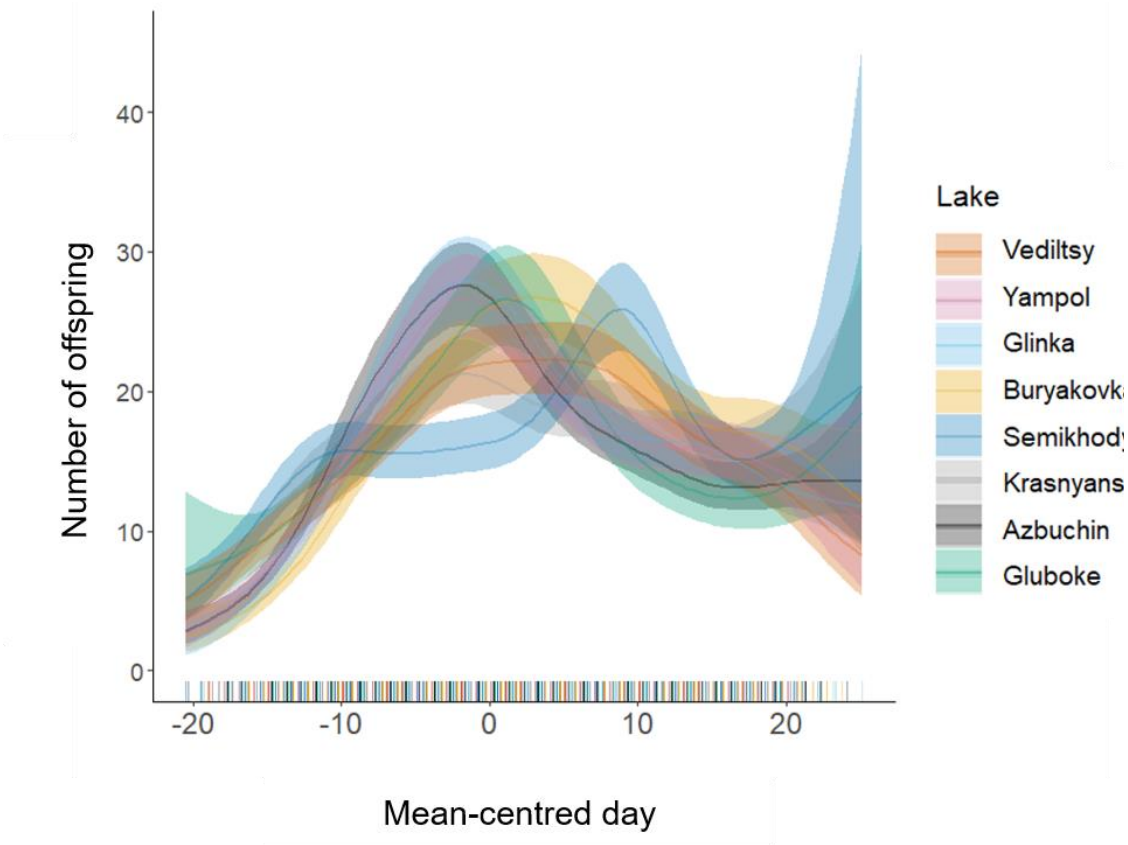


Figure 2.4: Age-specific reproduction according to Lake Population. The lines represent predictions based on a Generalised Additive Mixed Model (GAMM) fitting the number of offspring produced on each mean-centred day, smoothed by lake population. Jar nested within line nested within lake was fitted as a random effect. The shaded areas show 95% confidence intervals (CIs). The model was fitted using the *visreg* package (Breheny and Burchett, 2017).

2.4.3 Radiation exposure does not affect overall fitness

I found no effect of dose rate (GLS: $F_{1,29}$= 0.001, p= 0.98; Figure 2.5A) or lake population (GLS: $F_{7,23}$= 2.08, p= 0.09; Figure 2.5B) on *r*. Variation in *r* did not vary according to dose rate (χ^2_7= 2.58, p = 0.92; Figure 2.5A) or lake population (Bartlett's K^2_7= 4.97, p= 0.66; Figure 2.5B).

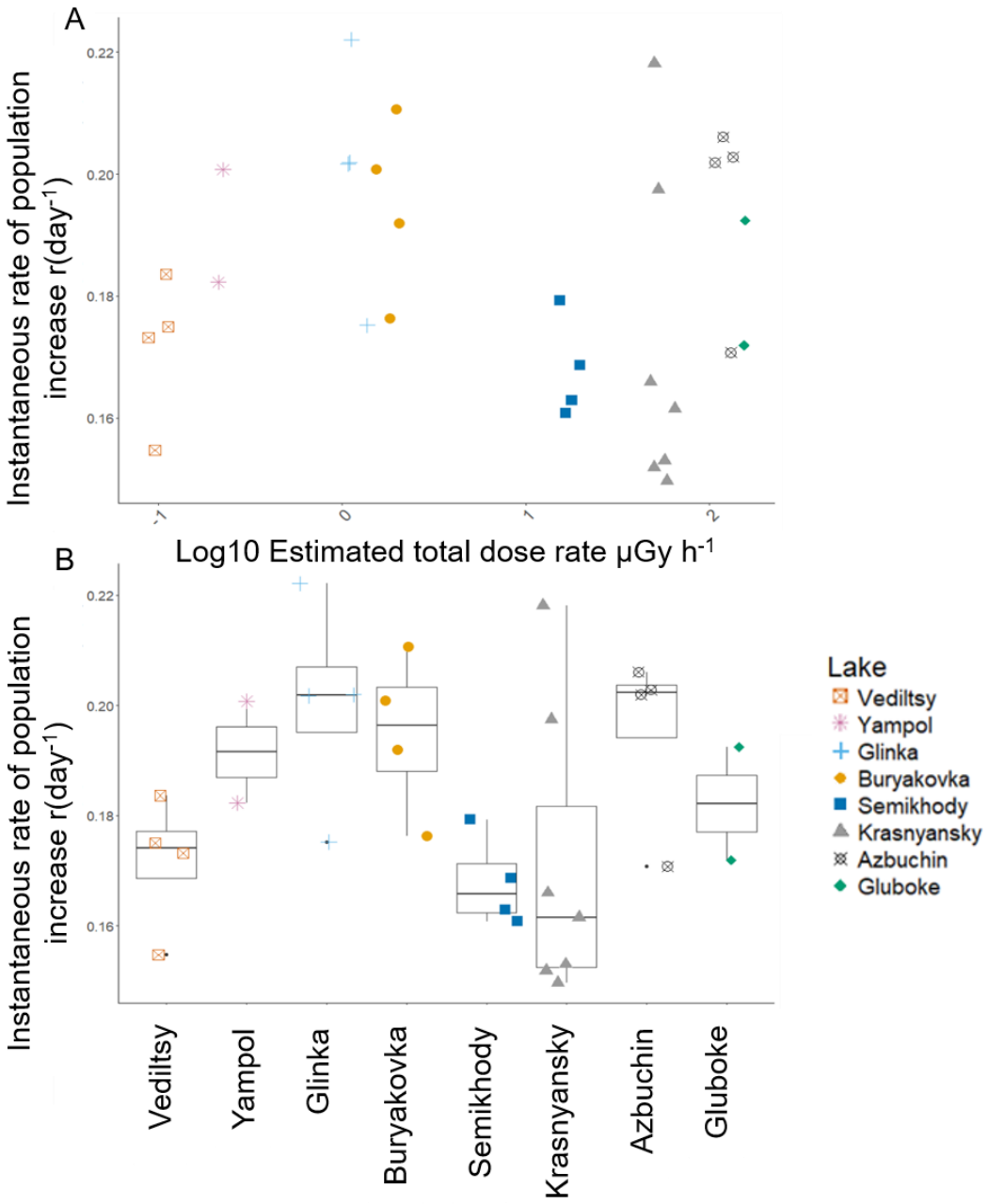


Figure 2.5: Boxplot showing the instantaneous rate of population increase *r* (day^{-1}) for each genotype within each lake population. Populations are plotted in order of increasing dose rate. The box shows the upper and lower quartiles within the data and the line within each box shows the median value. The lines outside of each box show the range of the data.

2.5 Discussion and conclusions

In this Chapter I presented the results of an experiment designed to examine variation in *Daphnia* survival and fecundity across populations in Chernobyl that have experienced very different levels of exposure to chronic radiation. I found no overall effect of dose rate on *Daphnia* survival. Laboratory-based studies have previously demonstrated that ionising radiation negatively affects invertebrate (including *Daphnia*) survival (Sarapultseva & Gorski, 2013; Nohara *et al.*, 2014; Parisot *et al.*, 2015). Parisot *et al*., (2015) found elevated mortality in *Daphnia* under radiation exposure, but only when animals were exposed for multiple generations under very high dose rates (4.7×10^3 µGy h^{-1} and 3.54×10^4 µGy h^{-1}) (Parisot *et al.*, 2015); these are much higher doses than those found in the CEZ, (I estimated ~180 µGy h^{-1} in Gluboke lake, which experienced the highest dose rate). However, this is not to say that exposure to radiation cannot affect natural populations: for example, CEZ populations have been exposed over a considerably longer period and to a variety of additional stressors that may confounding impacts (Holmstrup *et al.*, 2010).

After a careful and detailed examination of *Daphnia* reproduction - from total offspring output to subtle changes in reproductive investment through age-specific reproduction and proportion of non-reproducing individuals - I found no evidence of radiation-mediated effects. Variability in total offspring output was driven by lake Yampol (categorised as very low) only and each lake population had a unique pattern of offspring production with variable timing of peak reproduction, independent of dose rate. There is limited research on radiation-mediated life history shifts in wild populations; these studies found that irradiated groups invested in greater reproductive output but had similar overall population sizes. For example, due to differences in survival or reproductive schedules Blaylock (1969) Cooley (1973). The fact I find no effect of dose rate on *Daphnia* survival may explain why I observe no correlated effect on reproduction.

Whilst reproduction and survival provide valuable measures of fitness, the timing of reproductive investment with respect to lifespan is also important. The instantaneous rate of population increase (*r*) is a particularly useful measure, because it accounts for the fact that offspring produced in early life make a greater contribution to the mother's fitness than those produced later (Birch, 1948). I calculated *r* for each isofemale line and determined if mean or variance in *r* varied according to radiation dose rate. Specifically, I tested whether: (1) *r* declines and variation in *r* increases with dose rate, consistent with radiation-mediated supply of mutations reducing overall fitness whilst

increasing variation; or (2) that there would be no overall difference in mean *r* across populations, but variation in *r* would decline with increasing dose rate, consistent with stronger selection at higher radiation levels. Radiation dose rate was not associated with either the mean or variance in *r,* showing that historic radiation exposure is not the primary driver of variation in *Daphnia* fitness in these Chernobyl populations.

It is important to acknowledge that lack of association between radiation dose and life history variation at the population level does not mean that radiation is not having any effect. Radiation-mediated effects on reproduction within individual *Daphnia* have been demonstrated in the laboratory at dose rates as low as 7 µGy h^{-1} (Parisot *et al.*, 2015). However, in natural populations, a variety of ecological factors such as competition, predation or parasitism apply strong and often variable selection on populations (Brockelman, 1975; Lehmann, 1993; Creel and Christianson, 2008; Auld *et al.*, 2013). These ecological factors are therefore likely to be bigger drivers of life history variation than current dose rates. This brings into sharp focus the fact that few studies consider how the effects of radiation on individuals might scale to effects at the population or ecosystem level. A notable exception is a conceptual model by Polikarpov that predicts the negative effects of radiation on individuals will be overshadowed by much stronger interactions between the population and the wider ecosystem at higher radiation doses (termed "ecological masking"; Polikarpov, 1998). Notably, the estimated dose rates in this Chapter (~0.10 - 180 µGy h^{-1}) fall within those predicted to cause the “Ecological masking zone” in Polikarpov’s model.

I tested whether key life history traits varied across *Daphnia* populations that experienced a wide range of chronic radiation exposure in the Chernobyl Exclusion Zone. I found no such effects. It is clear that although radiation is known to negatively affect individuals, one needs to view it as one of many sources of selection in ecologically complex communities. Future research needs to widen the focus to other highly contaminated areas such as Fukushima (Saito *et al.*, 2015), and dissect the possible interactions between radiation and other stressors on individual fitness. The challenge now is to quantify the impacts of radiation relative to competition, predation, parasitism *etc.* in order to have a more complete understanding of the effects on radiation on the wider ecosystem.

Chapter three:

Evolution under ionising radiation: The genetic structure of *Daphnia* populations in Chernobyl

3.1 Abstract

Populations experiencing varying levels of ionising radiation provide an excellent opportunity to study the fundamental drivers of evolution. Radiation can supply mutations, and thus genetic variation; it can also select against individuals that are unable to cope with the physiological stresses associated with exposure to radiation. Since the nuclear power plant explosion in 1986, the Chernobyl area has experienced a spatially heterogeneous exposure to varying levels of ionising radiation. In this Chapter, I sampled *Daphnia pulex* (a freshwater crustacean) from lakes across the Chernobyl area, genotyped them at eleven microsatellite loci, and calculated the current radiation dose rates. I investigated whether the pattern of genetic diversity was consistent with either increasing levels of mutations, or increased selection pressure at higher dose rates. I found that measures of genetic diversity, including expected heterozygosity (an unbiased indicator of diversity) were significantly higher in lakes that experienced higher radiation dose rates; this is consistent with the hypothesis that there is higher mutational input at higher dose rates. I also found clear evidence for isolation by distance between populations, indicating that gene flow between nearby populations is breaking down population structure, and that mutational input in high radiation lakes could, ultimately, supply genetic variation to lower radiation sites. These evolutionary patterns can plausibly explain the lack of phenotypic variation associated with radiation dose rate in *Daphnia* in Chapter two.

3.2 Introduction

The explosion of the Chernobyl nuclear power plant in 1986 released approximately $1.85x10^{18}$ Bq of radionuclides into the atmosphere (IAEA, 2006a) that were subsequently deposited over the surrounding landscape (section 1.11). The deposition of this material was very heterogeneous, (Appendix A; Shestopalov, 1996), and wildlife populations in the Chernobyl area have thus experienced varying levels of radiation exposure depending on their locations (Hinton *et al*., 2007, Table 2.1); dose rates are also known to show considerable variation over very small spatial scales (Shestopalov, 1996). Whilst it is known that high doses of ionising radiation have strong negative effects on organismal fitness (IAEA, 1992; Barnthouse, 1995; Real *et al*., 2004) and can cause genetic mutations (Breimer, 1988; von Sonntag, 2007) that can be passed on to future generations (Adewoye *et al*., 2015), very little is known of how chronic exposure to radiation over multiple generations affects the population structure and genetic diversity (and thus long-term health) of populations.

Nuclear accidents like Chernobyl and Fukushima provide a window through which to view the three fundamental processes in evolutionary biology: mutation, selection and genetic drift (random changes in allele frequencies). Ionising radiation generates mutations (Breimer, 1988; von Sonntag, 2007) and can thus increase the supply of genetic variation to populations (Haldane, 1937; Kimura and Maruyama, 1966). This is important, because genetic diversity is the currency for both evolution and adaptation (Lande and Shannon, 1996). The dose rates across the CEZ have considerably declined since 1986, so if the current dose rates are sufficient to generate, radiation-mediated mutational supply, it will manifest as a positive relationship between measures of within-population genetic diversity and dose rate. Populations living within Chernobyl have been subject to long-term chronic exposures, which will have had a selective impact upon populations. This could lead to the removal of individuals with inadequate mechanisms for protecting against radiation-mediated cellular damage (Ramana *et al*., 1998; Khodarev *et al*., 2004; Diehn *et al*., 2009; Smirnov *et al*., 2012) and thus reducing genetic variation within and among high radiation exposed populations as a selective sweep (Schlotterer *et al*., 1997). Finally, the initial fallout from the accident could have caused bottlenecks across the whole area, depleting diversity and causing non-selective differentiation, *i.e.*, drift, among populations (Frankham *et al*., 2002); this would leave its mark in the form of strong population structure (Hartl and Clark, 1997), provided there was low gene flow (Slatkin, 1987; Gilpin, 1991).

In this Chapter, I used 11 microsatellite loci to examine the population genetic structure of the freshwater crustacean, *Daphnia pulex*, in seven *Daphnia*-inhabited lakes. Five of the lakes were within the Chernobyl Exclusion Zone (CEZ) and the other two were situated outside the CEZ. *Daphnia* are the ideal organism for such a study because of their habitat and cyclically parthenogenetic reproduction (Hebert, 1987; Colbourne *et al*., 2011). *Daphnia* populations are defined by the boundary of the water body that they inhabit and the heterogeneous nature of radionuclide deposition across the CEZ provides variation in radiation exposures that are independent of distance between populations (Shestopalov, 1996). In addition, migration between populations is limited to the dormant stage of sexual reproduction, where eggs (ephippia) can, on rare occasions, be transported between populations (Maguire, 1963; Alekseev and Lampert, 2001); this means gene flow is more restricted than in terrestrial species.

I explored whether genetic diversity increased with dose rate, consistent with increased mutations at more contaminated sites. This would manifest as a positive relationship between dose rate and some, or all of the genetic diversity parameters (observed and expected heterozygosity, the number of alleles, the number of private alleles and mean allelic richness) (Figure 3.1A). Alternatively, *Daphnia* living in more contaminated regions may undergo radiation-mediated selection to cope with radiation stress, which would result in a negative relationship between dose rate and measures of genetic diversity (Figure 3.1B). I also tested whether there was any evidence of genetic bottlenecks across populations, which would have resulted from the acute exposures from the Chernobyl disaster in 1986. This would be evidenced by low genetic diversity across populations and high population structure (assessed as the fixation index, F_{ST}; see also Figure 3.1C, which outlines a potential multivariate view of dose-driven population structure). I also examined the possibility that ecological factors other than radiation are having a greater influence on genetic variation across *Daphnia* populations, as discussed in Chapter two. This would be evident as significant isolation by distance (Figure 3.1D).

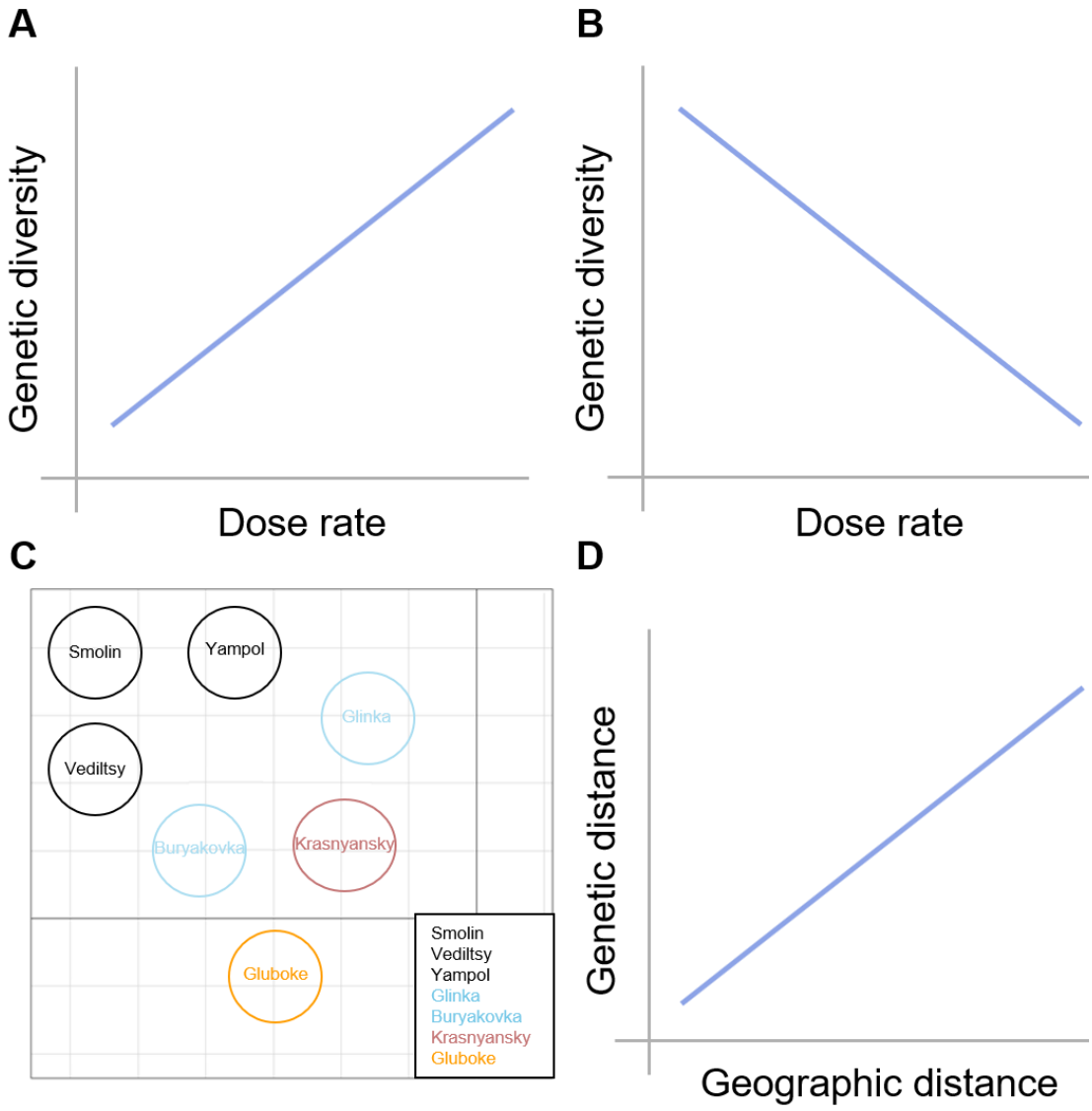


Figure 3.1: Diagrams representing the predicted outputs for the following hypothesis: (A) genetic diversity increases with dose rate consistent with mutational input, (B) genetic diversity decreases with dose rate in response to selection, (C) dose level drives differentiation among populations, consistent with genetic drift, (D) there is evidence for isolation by distance across populations. Note that in (C), black populations are very low dose, blue are low dose, red is intermediate dose and gold is the highest dose of radiation.

3.3 Materials and methods

3.3.1 Sample collection

Live *Daphnia*, sediment and water samples were collected from seven *Daphnia*-inhabited lakes within and immediately outside the CEZ from the 7th – 16th July 2016.

Individual daphnids were immediately stored in 1.5 mL Eppendorf tubes in absolute ethanol at ambient temperatures and transported back to the UK, where they were stored at -20 °C before genotyping. A total of 93 samples were collected from seven lake populations (12-22 samples per lake; see Table 3.2). Radiation dose rate was calculated according to the protocol detailed in section 2.3.2, where I measured/extracted the environmental activity concentrations of the different radionuclides at each site and used the values to estimate the dose rates to *Daphnia*. In brief, concentrations for the dominant radionuclides in the CEZ (^{137}Cs and ^{90}Sr) and radionuclides that were considered representative of others present within the water column and top sediment layer (^{241}Am and ^{239}Pu) (IAEA, 2006a) were used to estimate dose rates. Where information was available, data on radionuclide concentrations was extracted from the Ukraine atlas (Intelligence Systems GEO, 2008). Where information was unavailable, water and sediment samples were collected at each sampling site and analysed at the Ukrainian Hydrometeorological Institute (see section 2.3.2 for protocol).

To estimate dose rates experienced by *Daphnia*, I used the ERICA (version 1.2) software program. ERICA calculates dose rates to selected reference taxa based on information on radionuclide transfer through the environment and the activity concentrations given by the user (Beresford *et al.*, 2007; Brown *et al.*, 2008; ICRP, 2009). In this case, I selected Zooplankton as the reference taxon. As previously detailed in Chapter two, section 2.2, the occupancy parameters which describe the location of the organism in the water body were changed to 25% water column and 75% surface sediment to account for the dormant stages of the *Daphnia* lifecycle (Alekseev and Lampert, 2001). These percentages are conservative as the majority of radionuclides will accumulate in the surface sediment (Nazarov and Gudkov, 2009).

3.3.2 DNA extraction and Microsatellite genotyping

Microsatellite genotyping was used to identify differences in allele frequencies and population structure within and across lake populations following the protocol previously outlined by Auld and Brand (2017). First, genomic DNA was extracted from 93 whole organism *Daphnia* samples from seven lake populations (see Table 3.2 for details) using protocols provided in NucleoSpin Tissue XS (Machery Nagel). I successfully amplified eleven microsatellite markers for each *Daphnia* across two multiplexes (Table 3.1, Jansen *et al.*, 2011). Multiplex PCR reactions consisted of 5 µL 2 ×Type-it Multiplex PCR Mastermix (Qiagen), 3 µL Nuclease Free H_2O, 1 µL primer mix solution and 1 µL DNA to give a total volume of 10 µL per reaction. The PCR

programme was as follows: 15 minutes at 95 ºC for Taq activation, followed by 30 cycles of 30 seconds at 94 ºC for denaturation of the DNA into separate strands, 90 seconds at 57 ºC for annealing of the DNA strands to template DNA and 90 seconds at 72 ºC for extension. The final extension was performed for 30 minutes at 60 ºC. The final PCR products were analysed with an ABI 3730XL DNA Analyzer using the GeneScan-500 LIZ size standard (Applied Biosystems). Microsatellite band scoring was completed manually using GENEIOUS software (Biomatters, version 9.1.8). The strongest peak(s) within the loci were selected to determine allele size.

Table 3.1: Information for the 11 microsatellite loci used for genotyping *Daphnia* samples. M = multiplex.

Locus	Size range	NCBI accession number	Primer sequence (5'-3')	M	Dye label
B008	150 - 175	HQ234154	F: TGGGATCACAACGTTACACAA R: GCTGCTCGAGTCCTGAAATC	1	VIC
B030	150 - 176	HQ234160	F: CCAGCACACAAAGACGAA R: ACCATTTCTCTCCCCCAACT	1	PET
B050	229 - 248	HQ234170	F: TTTCAAAAATCGCTCCCATC R: TATGGCGTGGAATGTTTCAG	1	6FAM
B064	135 - 155	HQ234172	F: CTCCTTAGCAACCGAATCCA R: CAAACGCGTTCGATTAAAGA	1	6FAM
B065	323 - 408	HQ234173	F: AATCGCTCCCATCAACTCTG R: AGGCTCTCTTTCGTGTGAGG	2	6FAM
B174	330 - 375	HQ234205	F: CATATTGGCACGACGTTCAC R: GTTCCCTCATTCCCGATTTT	2	NED
B031	196 - 248	HQ234161	F: GTTGGCGCTGGCATATGTA R: AAGAATTTTTGCAGCCGTTG	2	6FAM
B075	124 - 150	HQ234175	F: GCTTGGGATCTCGAGAAGAA R: ACTTGCTAGTGGCTGCTGCT	2	PET
B088	155 - 170	HQ234179	F: GGACAGTCGGCGTTCACT R: CCTGTCGTGTTTTGATTTCCT	2	NED
B135	170 - 200	HQ234191	F: AAAGAGGGAGAATGTTGTTAGGC R: TAAGGAGGGGGAAAAAGTGG	2	VIC
B155	290 - 321	HQ234195	F: GCGCATATGCAACAATTCAC R: ACCTCCCCCTCACTTTGATT	2	PET

3.3.3 Analysis

The total number of alleles, mean allelic richness, the total number of private alleles, and observed and expected heterozygosities (respectively, H_O and H_E) were calculated (*PopGenReport* package; Adamack & Gruber, 2014, *adegenet* package; Jombart, 2008; Jombart and Ahmed, 2011). Kruskal-Wallis rank sum tests were then used to assess any differences in H_E, H_O, mean allelic richness, the total number of alleles and the total number of private alleles and across populations. Linear models were used to test the relationship between each of these measures of genetic diversity and $\log_{10}$ of the dose rate.

The number of multi-locus genotypes (MLGs) was determined (*poppr* package; Kamvar *et al*., 2014) and departures from Hardy-Weinberg equilibrium were assessed across loci (*pegas* package; Paradis, 2010). The index of unbiased association ($\bar{r}_D$; Brown *et al.*, 1980) was then determined in order to assess the level of linkage disequilibrium across populations; this was done using a permutation approach that estimates the levels of recombination in order to detect association between alleles (*poppr* package; Kamvar, Tabima and Grünwald, 2014).

Fixation indices (F-statistics) were then used to quantify population structure. Specifically the inbreeding coefficient (F_{IS}) and the fixation index (F_{ST}), were both calculated (*adegenet* package; Jombart, 2008; Jombart and Ahmed, 2011); these measure genetic differentiation within and among populations, respectively (Wright, 1951; Weir and Cockerham, 1984). Confidence intervals for the F_{IS} values for each population were computed by bootstrapping over loci, with 999 permutations using the *hierfstat* package (Goudet and Jombart, 2018). 999 permutations were used as it eliminates sufficient variation associated with resampling (Hesterberg *et al.*, 2003).

The next step was to test which populations were significantly different from each other. This was done by examining each pairwise F_{ST} comparison. To calculate the significance for each pairwise F_{ST} comparison, 999 permutations were used to randomly allocate populations and recalculate F_{ST} values to get a full reference distribution (*ade4* package, which uses Monte Carlo simulations, Dray & Dufour, 2007; Bougeard & Dray, 2018). These values were then compared to the observed values to calculate a p-value for each F_{ST} comparison. This approach was also used to compare F_{ST} values when populations were grouped according to radiation exposure level (Chapter one, Box 1). I then tested whether populations in close proximity to each other were more similar than those separated by larger geographic distances (*i.e.,* whether there was isolation by distance). This was done using a Mantel test, which tested for an

association between two matrices of pairwise Edward's genetic distances (Edwards, 1971) and pairwise Euclidean geographic distances between populations. Finally, an analysis of molecular variance (AMOVA) was used to partition variation within and between populations; the significance of these within- and among-population variation was then estimated using 999 permutations (*ade4* package; Dray & Dufour, 2007; Bougeard & Dray, 2018).

3.4 Results

3.4.1 Genetic diversity and dose rate

Estimated dose rates are given in Table 3.2 (see Appendix C for activity concentrations used to calculate dose rates in Smolin lake, located in Slavutych outside of the CEZ as Smolin was not included in Chapter two). There was a significant effect of dose rate on H_E ($F_{1,5}$= 7.01, p< 0.05. Figure 3.2A), but not of population on H_E (population: χ^2_6= 6.00, p= 0.42). There was no effect of either population or dose rate on H_O (population: χ^2_6= 6.00, p= 0.42, dose rate: $F_{1,5}$= 1.32, p= 0.30. Table 3.2, Figure 3.2B). Mean allelic richness did not vary across populations (χ^2_6= 6.00, p= 0.42), but showed a marginally non-significant positive relationship with dose rate ($F_{1,5}$= 5.91, p= 0.06. Figure 3.2C). The number of alleles per locus ranged from 29 to 45. Neither population or dose rate significantly affected the number of alleles (population: χ^2_6= 6, p= 0.42, dose rate: $F_{1,5}$= 4.28, p= 0.09), or the total number of private alleles (population: χ^2_6= 6, p= 0.42, dose rate: $F_{1,5}$= 0.07, p= 0.80).

Table 3.2: Estimates of genetic diversity among seven *Daphnia* populations at 11 microsatellite loci across Chernobyl.

Lake	Sampling date	Coord N	Coord E	Upper dose estimate	n	MLG	MLG/n	H_E	H_O	A	PA	MAR	F_{IS}	$\bar{r}_D$
Smolin	07.06.2016	51.2757	31.0333	0.12	12	12	1	0.57	0.45	39	2	2.68	0.21	0.02
Vediltsy	07.06.2016	51.4352	30.8385	0.1	12	12	1	0.47	0.53	29	0	2.26	-0.12	0.04
Yampol	11.06.2016	51.2095	30.1767	0.2	12	12	1	0.48	0.35	30	1	2.3	0.24	**0.07**
Glinka	16.06.2016	51.2174	29.9371	1.17	12	12	1	0.50	0.31	31	1	2.41	**0.34**	0.07
Buryakovka	11.06.2016	51.3978	29.8931	1.77	12	12	1	0.55	0.34	34	0	2.60	**0.3**	**0.25**
Krasnyansky	13.06.2016	51.4429	30.0764	55.79	24	24	1	0.65	0.5	45	3	3.06	**0.19**	**0.16**
Gluboke	13.06.2016	51.4454	30.0653	181.15	12	11	0.92	0.60	0.59	39	0	2.74	-0.02	0.06

(n) number of individuals, (MLG) multilocus genotypes, (HE) expected heterozygosity, (HO) observed heterozygosity, (A) number of alleles, (PA) number of private alleles, (MAR) meal allelic richness). Upper dose estimate is in µGy h^{-1}.

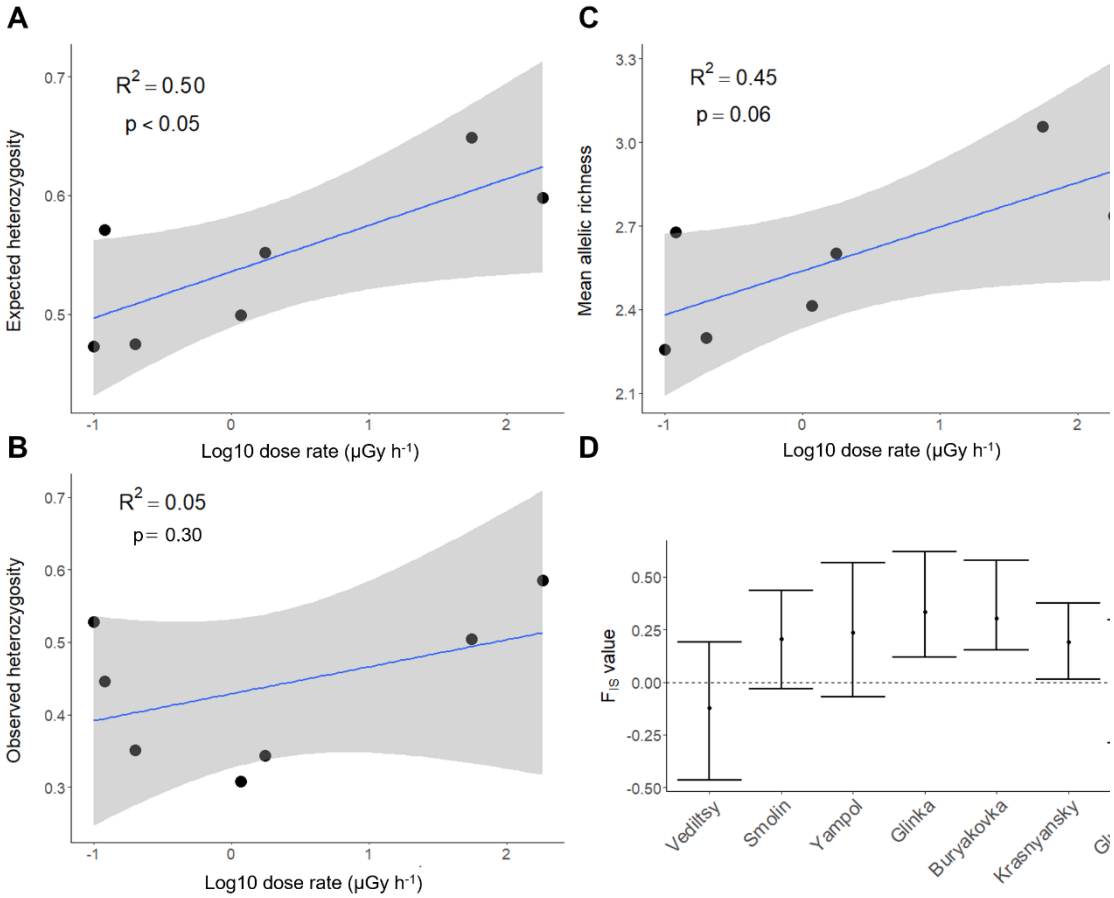


Figure 3.2: (A) The relationship between expected heterozygosity and log_{10} dose rate, (B) The relationship between observed heterozygosity and log_{10} dose rate, (C) the relationship between mean allelic richness and log_{10} dose rate. Points for figures A, B and C show the raw data and the shaded area shows 95% confidence intervals. The R^2 value is indicated for each model fit. (D) F_{IS} values and confidence intervals (computed using Monte Carlo simulations with 999 permutations) by population.

3.4.2 Hardy–Weinberg and linkage disequilibrium

I identified 92 multilocus genotypes (MLGs, Table 3.2) among the 93 individuals. The replicate genotype was removed from subsequent analysis as this was indicative of a clone (as a result of parthenogenetic reproduction). Observed heterozygosity ranged from 0.31 to 0.58 and expected heterozygosity ranged from 0.47 to 0.65 (Table 3.2). There were significant departures from Hardy-Weinberg equilibrium across the majority of loci (10 out of 11 loci, see Appendix D) and significant linkage disequilibrium was found in Yampol, Buryakovka and Krasnyansky lake populations (see Table 3.2).

3.4.3 Population structure and gene flow

The F_{IS} values ranged from -0.12 and 0.34 across lake populations (Table 3.2), but were significantly greater than zero (*i.e.,* indicative of inbreeding) in only three populations, Glinka, Buryakovka and Krasnyansky (Figure 3.2D). Overall population structuring was low for *Daphnia* (Thielsch *et al.*, 2009), where overall F_{ST} was 0.09 (Figures 3.3 and 3.4A). There were no significant differences in pairwise F_{ST} values between Yampol and Vediltsy populations ($p= 0.17$. Figure 3.3), or Gluboke and Krasnyansky ($p= 0.23$). All other pairwise F_{ST} values were significant ($p< 0.05$). Pairwise comparison of F_{ST} by radiation exposure level (see Chapter one, Box 1) showed lower overall structuring than when grouped by population (F_{ST} by exposure level = 0.05, Figure 3.4B). All pairwise comparisons of F_{ST} by exposure levels showed significant differences ($p< 0.05$). There was a significant relationship between genetic and geographical distances, *i.e.,* isolation by distance ($p< 0.001$. Figure 3.5).

An AMOVA found that there was significant variation within populations ($p< 0.01$, Table 3.3) and between populations ($p< 0.01$), confirming that there was significant population structure.

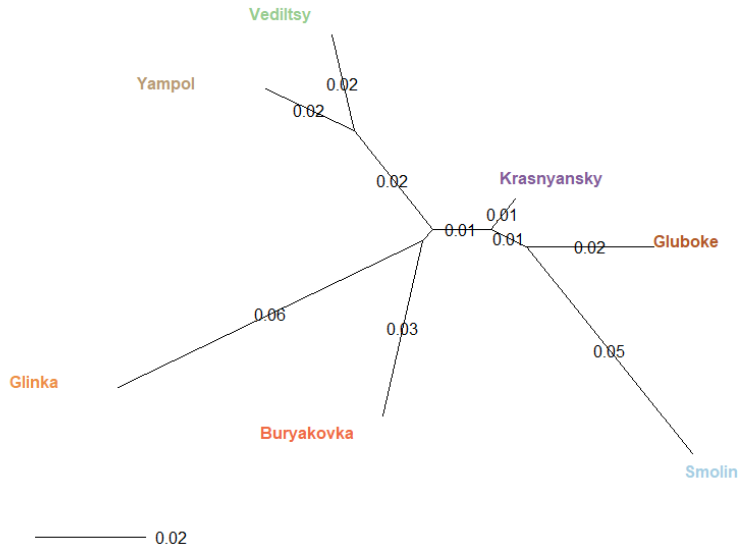


Figure 3.3: Genetic distance tree generated based on pairwise F_{ST} values using the *ape* package in R (Paradis and Schliep, 2018). The scale bar shows the scale of the branch lengths.

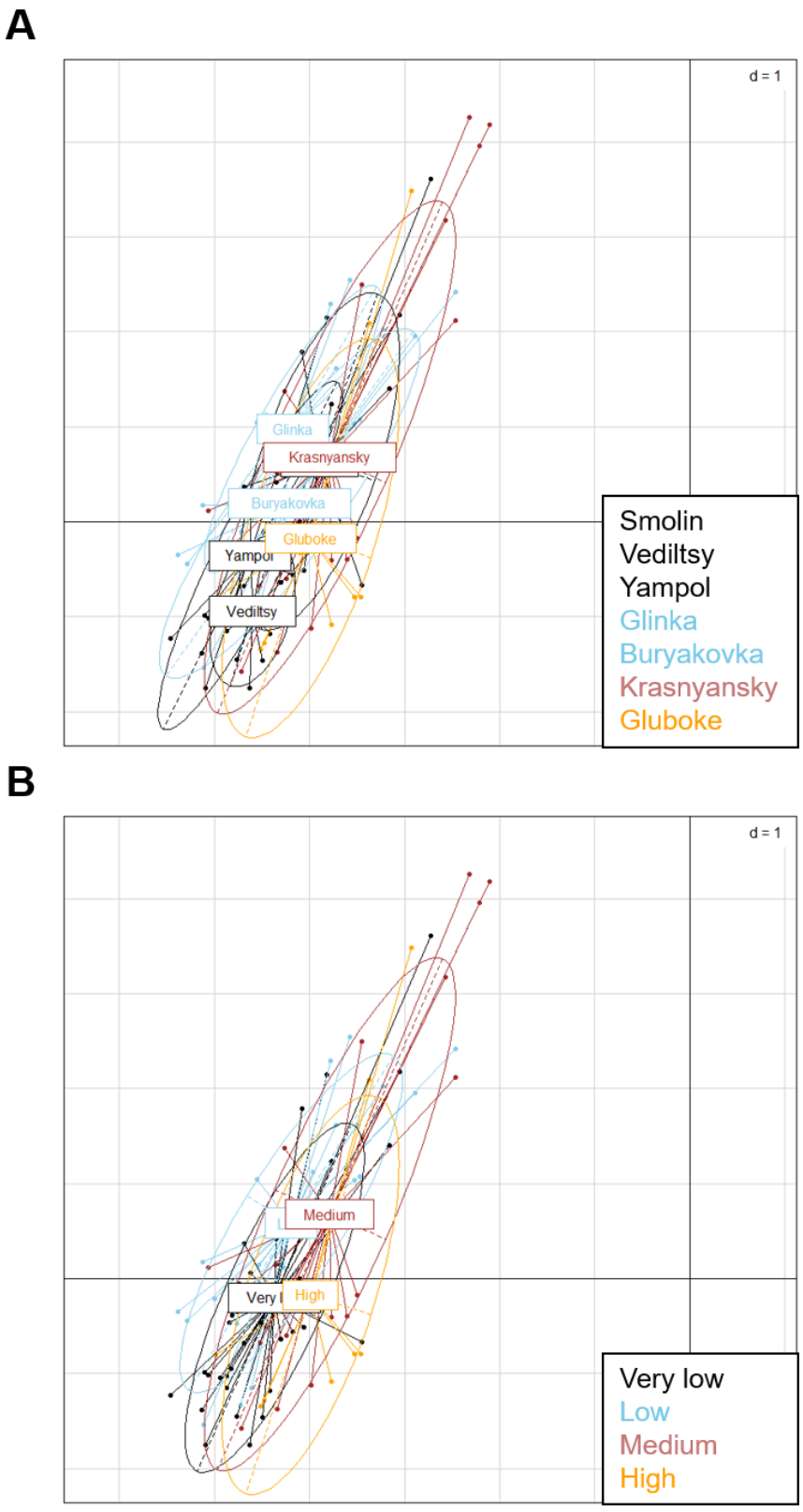


Figure 3.4: Principal component analysis (PCA) plots of the microsatellite frequency data. (A) Principal components based on microsatellite data from the seven lake populations specified in Table 3.2. (B) Principal components based on microsatellite data grouped according to the levels of exposure specified in Chapter one, Box 1.

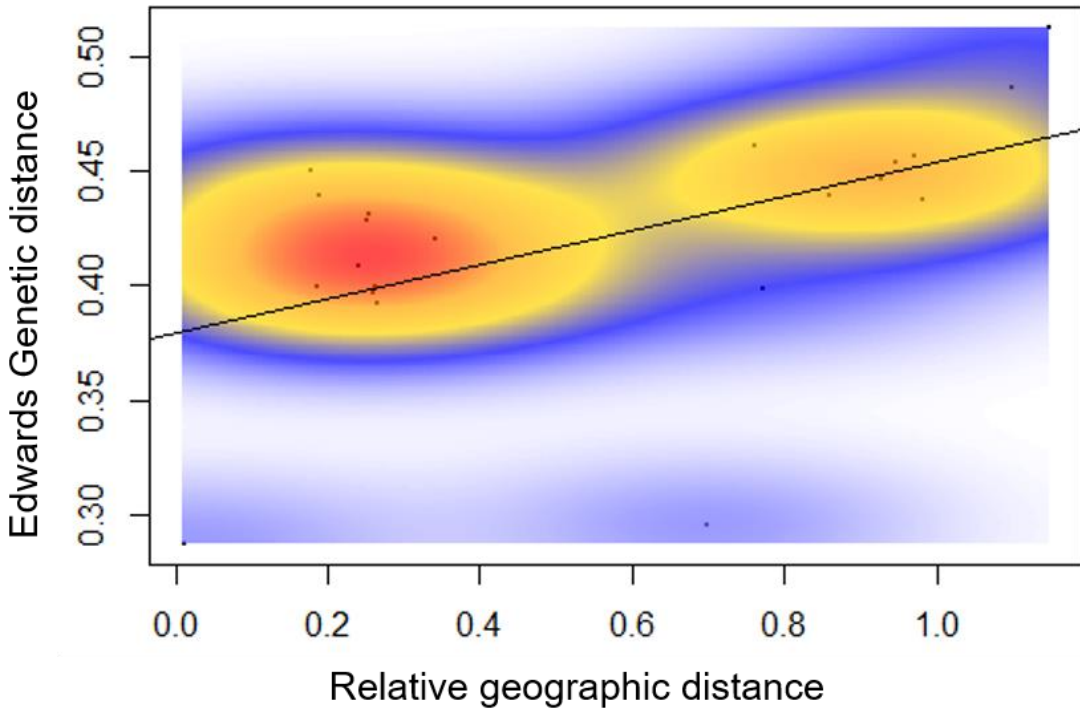


Figure 3.5: Isolation by distance plot based upon a Monte Carlo simulation using 999 permutations to test between two matrices of pairwise Edward's genetic distances and Euclidean geographic distances (see Appendix E for relative distance estimates). The heatmap shows the data distributions using local density estimations using a two-dimensional kernel density estimation, implemented using the *MASS* package (Venables and Ripley, 2002).

Table 3.3: Analysis of molecular variance (AMOVA) assessing the partitioning of genetic variation. Significant values are highlighted in **bold**.

Source of variation	**df**	**Sum of squares**	**Variance**	**% total**	***p***
Between populations	6	27.16	0.2	9.43	**0.001**
Within populations	86	164.52	0.191	90.57	**0.007**
Total	92	191.67	2.11	100	**0.001**

3.5 Discussion and conclusions

Ionising radiation supplies mutations and thus genetic variation to populations *(*Adewoye *et al* 2015; Breimer, 1988). This genetic variation is a substrate for evolution and potentially adaptation within populations (Muller, 1927; Haldane, 1937; Kimura and Maruyama, 1966). Radiation can also select against genotypes that are unable to cope with high radiation environments (Courtenay, 1965; Møller, 1993, 2002; Shestopalov, 1996; Ellegren *et al*., 1997), and thus deplete variation. I previously found that there was no evidence for phenotypic fitness differences due to radiation exposure in *Daphnia* populations across the CEZ (Chapter two; Goodman *et al.,* 2019). However, this does not mean radiation has no effect: evolutionary processes, such as radiation-mediated selection, could continually eliminate phenotypic differences among populations, reduce genetic variation and thus limit the potential for adaptation in response to future selection (Lande, 1993). In this Chapter, I tested whether variation in exposure to ionising radiation was associated with increased genetic variation within populations, signatures of radiation-mediated selection, and genetic structure among populations.

These data provide evidence consistent with radiation-mediated supply of genetic variation in wild populations (Geras'kin and Volkova, 2014). Higher doses of radiation are associated with increased genetic variation to populations, consistent with increased *de novo* mutational supply. This manifested as significant relationship between dose rate and expected heterozygosity across eleven microsatellite loci (despite having only examined seven populations). I also found a marginally non-significant ($p = 0.06$) positive association between dose rate and mean allelic richness. Other studies have revealed significantly higher mutation rates in microsatellite loci from samples within the CEZ experiencing contamination from the Chernobyl accident compared to local control sites (Dubrova *et al.*, 1996; Ellegren *et al.*, 1997; Kovalchuk *et al.*, 2000), even at very low dose rates (approximately 8.60 μGy h^{-1}, Kovalchuk *et al.*, 2003).

Microsatellites are neutral markers (Li *et al.*, 2002), whereas selection acts directly on functional genes. As such, one must be careful not to over-interpret patterns of selection using microsatellites. Nevertheless, since most mutations are deleterious, it is highly likely that radiation-mediated selection, and thus genetic load, also correlates with the radiation dose experienced by populations. Any long-term directional selection associated with chronic exposure would deplete genetic variation (Mort and Wolf, 1986) as genotypes with poor anti-stress mechanisms were removed from high but not

low dose populations (Ramana *et al.*, 1998; Khodarev *et al.*, 2004; Diehn *et al.*, 2009). The data in this chapter are consistent with the hypothesis that mutational supply outweighs any radiation-mediated depletion of genetic variation in these Chernobyl *Daphnia* populations.

Additionally, I tested whether the Chernobyl accident may have indiscriminately culled genetic diversity within populations, driving genetic drift (Frankham *et al*., 2004) and reducing the effective population size (Nei and Tajima, 1981). This is crucially important, as when effective population sizes are low, both beneficial and deleterious mutations are effectively neutral (Hartl and Clark, 1997), and there is little capacity for selection to drive adaptive evolutionary change (Lande, 1993; Hartl and Clark, 1997). As well as low diversity, genetic drift leads to greater differentiation among populations and strong population structure. There is little evidence for either: as discussed earlier, genetic diversity is surprisingly high and correlated with radiation dose, and measures of inter-population differentiation (pairwise F_{ST} values) are low (0.03-0.13: Appendix D). Further, the overall F_{ST} (summary measure of population structure) and is also low, and over 90% of the overall genetic variation is due to within-population differentiation and individuals do not cluster according to population (see Figure 3.4 and Appendix F). These results provide strong evidence that genetic drift is not a strong force among Chernobyl *Daphnia* populations.

This Chapter uncovered evidence of inbreeding in three of the populations (at low / medium dose rates), and no evidence for heterozygote excess. This is in stark contrast to the related *Daphnia magna,* where heterozygote excess is the norm and systematic inbreeding is either rare or completely absent (Hebert, 1974a, 1974b; Hebert and Ward, 1976; Haag *et al.*, 2006; Walser and Haag, 2012). One possible reason for my findings is that the sex ratios varied among lake populations. The production of males in *Daphnia* populations is known to be determined by environmental change (such as increased population density, light levels or high levels of toxins) (Hobaek and Larsson, 1990; Eads, Andrews and Colbourne, 2008). Biased sex ratios are known to cause inbreeding, particularly in small populations (Mills and Smouse, 1994). Radiation is unlikely to be driving this, as inbreeding was not clearly linked to higher dose rates, suggesting that alternative ecological factors are responsible for inbreeding effects.

It is important to consider the complex reproductive biology of *Daphnia* when assessing MLGs. Genetic recombination is followed by a period of asexual reproduction and asexual reproduction is often accompanied by clonal selection, where selection on any one trait involves selection on the whole genome (Lynch, 1987). Clonal selection

means the same MLG is represented in multiple individuals (Halkett, 2005), F_{IS} values become negative within years as heterozygotes become overrepresented in the population and linkage disequilibrium can accumulate (this is in contrast to selection acting across bouts of sexual reproduction, which could generate positive F_{IS} values). I find only one instance, in Gluboke lake, where the same MLG was collected twice and, as discussed earlier, there are no significantly negative F_{IS} values for that population. This could be because the sampled populations are sufficiently large to host huge *Daphnia* populations where the frequency of sex is high (Allen and Lynch, 2012). Therefore, the sampled populations are effectively behaving as if they were purely sexual. However, I do find evidence of past linkage disequilibrium ($\bar{r}_D$) and thus past clonal selection in Yampol, Buryakovka and Krasnyansky lakes. The strength of this past linkage disequilibrium is not, however, associated with dose rate, suggesting that past bouts of clonal selection are caused by other biotic/abiotic conditions.

Finally, high radiation environments could potentially supply genetic variation to other nearby populations as *Daphnia* resting stages disperse, fuelling within-population evolution and adaptation (metapopulations, Hanski, 1998). I uncovered evidence, in the form of strong isolation by distance, that gene flow from dispersal from neighbouring populations is reducing levels of population structure. As such, it is likely that the lack of phenotypic variation among Chernobyl *Daphnia* populations (Goodman *et al.* 2019*;* Chapter 2) is concealing highly dynamic demographic and evolutionary processes that are associated with ionising radiation.

Chapter four:

How *Daphnia pulex* respond to Chernobyl relevant dose rates in a controlled irradiation facility, including the dose characterisation of the irradiation facility at Stirling University

4.1 Abstract

Dose estimates made for wild populations living within high radiation environments are often complicated due to factors such as ingestion of additional radioactive material and movement across a heterogenous landscape of contamination, and inadequate dosimetry approaches can lead to false conclusions on radiation effects. Dose response experiments under controlled laboratory conditions therefore provide useful data for dissecting radiation responses at accurately measured dose rates, thus supporting field observations. In this Chapter, I characterise the irradiation facility at the University of Stirling by first using the Monte Carlo Code for Neutron and Photon Transport (MCNP) to simulate dose rates across the facility, followed by verification of actual dose rates using Thermoluminescent dosimeters (TLDs) and Electronic Personal Dosimeters (EPDs). I then assess radiation responses in a *Daphnia* genotype sampled locally to the Chernobyl Exclusion Zone (Slavutych, situated approximately 50 km from the Exclusion Zone) to dose rates relevant to Chernobyl exposures (0.45 – 350 µGy h^{-1}, compared to estimated Chernobyl dose rates to *Daphnia* <~ 180 µGy h^{-1}). I found that the MCNP simulations are consistent with dose rates measured using both the TLDs and EPDs. I then found a significant relationship between the total number of offspring produced and dose rate. There were no significant radiation effects across any other measures of *Daphnia* fitness (survival, time until first brood, age-specific reproduction). The subtle, sublethal nature of these results provide justification for using a single high dose rate treatment in the following Chapter, which explores transgenerational radiation effects.

4.2 Introduction

Radiation exposure from the Chernobyl disaster can be separated into acute and chronic phases (Hinton *et al.*, 2007). Whilst there is an abundance of research testing the biological effects of acute radiation exposure, there are still substantial knowledge gaps on the effects of chronic exposure at environmentally relevant doses, for example, within the Chernobyl Exclusion Zone (CEZ), (Copplestone *et al*., 2008; Salbu, 2009; Fuller *et al.*, 2015). Furthermore, controversies exist surrounding some of the conclusions made in the existing literature for chronic field exposures, where data show substantial negative biological impacts at very low reported dose rates (in some cases close to, or below, natural background dose rates seen in the UK) (Section 1.8, Møller and Mousseau, 2009, 2011; Mousseau and Møller, 2011). These studies are at odds with the literature from laboratory studies and some other field studies (Beresford and Copplestone, 2011; Garnier-Laplace *et al.*, 2013). It is important to resolve these controversies in order to reduce uncertainty in our general understanding of radiation induced effects and also for establishing robust radiation benchmarks for wildlife for use in risk assessment. Laboratory-based studies can provide useful verification for results found in the field (Bréchignac *et al.*, 2016) and address some of the uncertainty involving different dosimetry approaches used for field populations (Garnier-Laplace *et al.*, 2013).

Radiation impacts have been shown to transmit across generations, having long lasting lineage effects (Dubrova, 2003; Morgan, 2003a, 2003b) and, as radionuclides are continually decaying in the CEZ, it can therefore be difficult to be certain that observed biological impacts in the field are a direct result of current radiation doses, rather than an effect of historical radiation doses. For example such as those experienced in the immediate aftermath of the accident in 1986 (Omar-Nazir *et al.*, 2018). Laboratory studies can help to elucidate this.

Testing radiation effects in isolation also removes the influence of confounding factors, which may have synergistic or antagonistic effects with radiation, thereby exacerbating or reducing observed biological effects (Burkart *et al*., 1997; Folt *et al.*, 1999; Vanhoudt *et al.*, 2012). Conducting dose response experiments at environmentally relevant dose rates (*i.e.* dose rates ranging from natural background to those observed in the worst affected areas of accidental releases such as at Chernobyl and Fukushima) also bypasses extrapolation errors commonly associated with predicting chronic exposure impacts based on the results of acute high level exposures usually seen in laboratory

studies (IAEA, 2006b; Dauer *et al.*, 2010; Garnier-Laplace *et al.*, 2013; Mothersill *et al.*, 2019).

Careful consideration needs to be given to the levels of biological organisation being studied, especially as laboratory studies generally focus on individuals whereas field assessments usually test populations. Individual assessments are likely to lose predictive reliability at the population level in the field due to a variety of factors, including sub-optimal conditions, multiple stressors in natural environments and population processes such as adaptation and selection (Bréchignac, 2003; Esnault, Legue and Chenal, 2010; Galván *et al.*, 2014; Spurgeon, 2018). In addition, laboratory studies are often limited to assessment of a single generation, whereas wild populations in high radiation environments such as the CEZ will be exposed across multiple generations, resulting in the potential accumulation of effects. This includes effects such as genomic instability (Section 1.5; Mothersill and Seymour, 1998; Dubrova, 2003; Morgan, 2003a, 2003b; Barber *et al.*, 2006). It is therefore important to remain aware of the factors that may compromise extrapolation to reduce uncertainty in estimating risk to wildlife (Spurgeon, 2018).

This chapter describes the irradiation facility at Stirling University that was used to conduct the laboratory exposure experiments within this project. First, the irradiation facility at Stirling University was characterised by verifying the dose rates across the experimental area. Dose rates across the irradiation facility were predicted using the Monte Carlo Code for Neutron and Photon Transport (MCNP). MCNP is a general purpose code for simulating neutron, photon, and electron transport under specified conditions (Briesmeister, 1986; Hendricks *et al.*, 2000). Different time dependencies and 3-Dimensional geometries can be specified to accurately predict dose rates for a variety of applications, including for medical purposes and environmental risk assessment (Whalen *et al.*, 1991; Kiger *et al.*, 2005; Szoke *et al.*, 2014). These predicted dose rates were then verified using both Electronic Personal Dosimeters (EPDs) and thermoluminescent dosimeters (TLDs) to measure exact dose rates at different points throughout the irradiation facility. The same approach was also applied to assess the shielding effects of the glass jars containing *Daphnia* media that contained each experimental *Daphnia*.

Second, preliminary data were generated on the effects of environmentally relevant dose rates to *Daphnia* under controlled laboratory conditions (in the context of the CEZ, dose rates to *Daphnia* estimated in chapter two ranged from ~ 0.10 – 180 μGy h^{-1}), to inform the experimental design for a multigenerational assessment in Chapter five.

Literature examining radiation effects at dose rates relevant to those in the CEZ is scarce, however, one study by Parisot *et al*. (2015) revealed reproductive delays in *Daphnia* at dose rates as low as 7 µGy h^{-1} and no mortality within the ranges tested within the current Chapter (Parisot, *et al*., 2015). I hypothesise that there will be some negative effects on *Daphnia* reproduction (such as reproductive delays and a reduction in the total number of offspring produced), but no detrimental effects on survival. If this is the case across all examined dose rates, then I can be confident that *Daphnia* can survive to produce further generations in the following Chapter which will look at any potential transgenerational effects of radiation exposure.

I hypothesise that there will be some negative effects on *Daphnia* reproduction associated with increased radiation dose rate, which should be evidenced by a reduction in the total number of offspring produced, delays in reproduction and negative effects on age-specific fecundity (Figures 4.1A, 4.1B and 4.1C). I predict that there will be no detrimental effects on survival, demonstrated by no difference in survival rate across treatment groups (Figure 4.1D). If this is the case across all examined dose rates, then I can be confident that *Daphnia* can survive to produce further generations in the following chapter which will look at any potential transgenerational effects of radiation exposure.

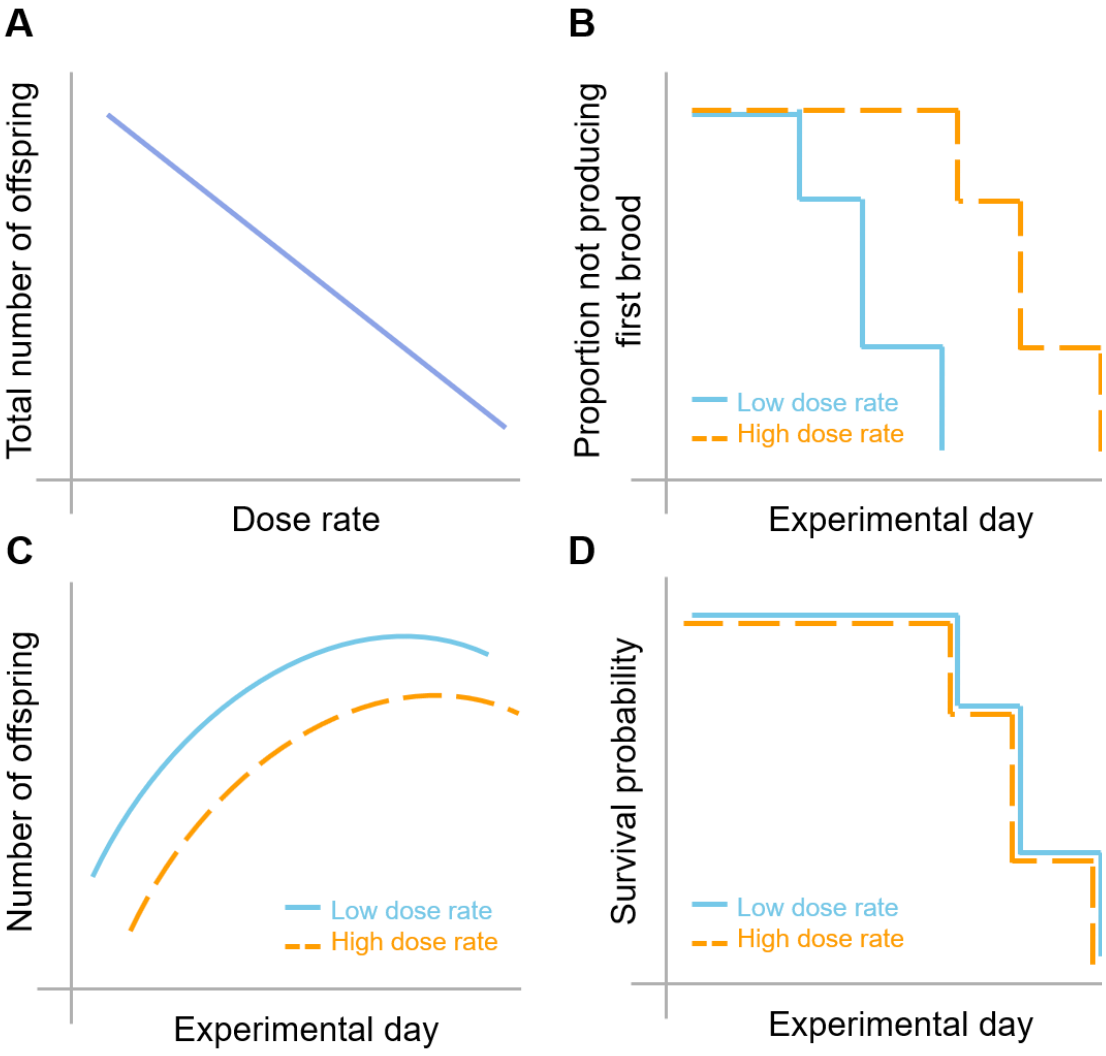


Figure 4.1: Example figures that show predicted results. (A) Negative effects of radiation dose rate on the total number of offspring produced, (B) delays in reproduction under a high dose rate treatment compared to a low dose rate treatment, (C) negative effects of dose rate on age-specific reproduction and (D) no effects of radiation dose rate on survival.

4.3 Materials and methods

4.3.1 Dosimetry

The irradiation facility at the University of Stirling (UoS) is built around an 18 GBq ^{137}Cs sealed source and is equipped with a temperature and light control system. The ^{137}Cs source (henceforth, source) is contained within lead shielding and operated via a computer within the instrument room. This moves the source from the active to the safe position with a radiation monitor showing when the source is exposed.

Characterisation of radiation dose was conducted using three approaches:

(1) Initial characterisation of the irradiation facility was conducted using the Monte Carlo Code for Neutron and Photon Transport (Briesmeister, 1986). Monte Carlo approaches are used to estimate particle transport based upon simulations of linear accelerators (Rogers *et al.*, 1995). The MCNP approach accounted for the structure of the source housing, as well as different distances from the source where the experimental units were placed throughout the facility (Brown, 2003). Each experimental unit consisted of a 50 mL glass jar containing *Daphnia* media (ADaM, Klüttgen *et al.*, 1994) and a single daphnid, so MCNP code was also used to estimate the shielding effect for each experimental unit.

(2) Thermoluminescent dosimeters (TLDs) were placed throughout the irradiation facility to verify the dose received. TLDs were attached to the front and back of 12 jars containing media to assess the shielding effects for each experimental unit for comparison to MCNP predictions.

(3) Electronic Personal Dosimeters (EPDs) were placed at each treatment group position to verify the dose received. The EPDs were used to verify that all jars within a given treatment group received the same dose rate. This was required because as the distance from the source increases, the position of treatment groups need to be at less of an angle to achieve a uniform radiation dose rate (Figure 4.2).

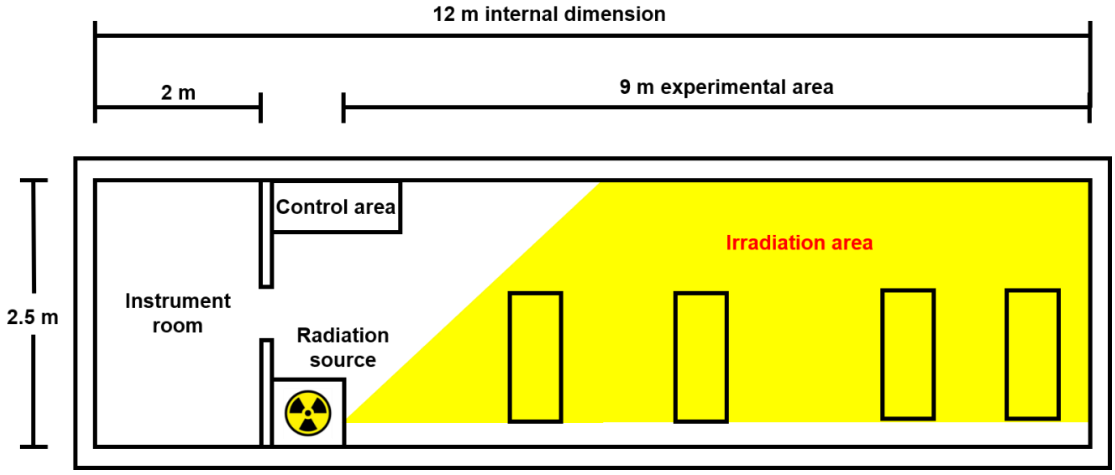


Figure 4.2: Illustration of radiation pathway through the irradiation facility from the ^{137}Cs source.

4.3.2 Study system

The *Daphnia pulex* genotype used for this experiment was taken from Lake Vediltsy situated in Slavutych, just outside of the CEZ (see Chapter two, section 3.2 for details). The estimated dose rate range that *Daphnia* in Vediltsy lake were exposed to at the time of collection in June 2016 was 0.07 – 0.1 µGy h^{1}, which is comparable to the highest average UK background doses at 0.18 µGy h^{-1} (RIMNET, 2017). Genotypes from Vediltsy lake have not recently been exposed to high radiation levels although they are situated close to the CEZ. Due to the proximity to the CEZ, their response is more likely to be representative of Chernobyl *Daphnia* populations than that of naive genotypes sourced elsewhere, such as in the UK. Using a genotype from Vediltsy therefore provides the best representation of whether the current dose rates seen in the CEZ could affect a wild contemporary population.

4.3.3 Dose response experimental setup

Based upon the results from the MCNP modelling approach, five dose rate treatments including the control treatment were selected for the dose response experiment (Table 4.1).

Five maternal lines of the selected genotype were setup to ensure that there were enough replicates to supply the experiment. Offspring from the third brood release of the maternal lines were randomly assigned to jars to ensure that there was no bias in the exposed and control treatments. All genotypes were setup on the same day (experimental day 1), so setup day was not a factor in experimental analysis. Twenty-five replicates from each maternal line were randomly assigned to the control and each

of four exposure treatments, giving a total of 125 experimental units across the whole experiment).

Daphnia media was replaced three times weekly or when broods were released. *Daphnia* were fed 1.0 ABS *Chlorella vulgaris* algal cells daily (where ABS is the optical absorbance 650 nm white light). Assessment of phenotypic traits, survival and reproduction was conducted daily for each individual. Reproduction was recorded for eight brood releases per individual and recorded as the number of offspring produced in each brood and the day of brood release. Survival was measured by recording the day of death for each individual.

4.3.4 Statistical analysis

All analysis of the dose response data was conducted using R statistical software version 3.4.3 (R Core Team, 2017). Summary data for this Chapter are included in Appendix G. First, the effect of radiation dose rate on the total number of offspring produced was tested using a generalised linear model (GLM), assessing the total number of offspring produced against dose rate. A Poisson distribution was used because count data were being analysed. Not all individuals assessed during the experiment produced all eight of the broods measured (for example, due to death), so the data were re-examined without these individuals included.

Secondly, the effects of radiation dose rate on the time taken until first brood release was assessed using a Cox's proportional hazard (CPH) model with day of first brood release as the survival object. This was conducted using the *survival* package (Therneau, 2015). Age-specific reproduction was assessed using linear mixed effects (LME) models to assess whether dose rate impacted the number of offspring produced on each experimental day, implemented using the *lme4* package (Bates *et al.*, 2015). Experimental days were mean-centred to enable testing of both the linear component (mean-centred day) and polynomial component (mean-centred day squared) of age-specific fecundity; dose rate and various two-way interactions were fitted as fixed effects. A random regression term was included for each replicate, with experimental day as the intercept to allow the slope predictions to vary for each individual replicate. The models assessed the number of offspring produced on a given day, measuring dose rate as a fixed effect. The significance of factors included within the LME model were determined using Type II sums of squares assessments (using the *Anova* function within the car package) (Fox and Weisberg, 2019).The effects of radiation dose rate on *Daphnia* survival were measured by fitting a CPH model to the survival data, with the day of death for each individual as the survival object.

4.4 Results

4.4.1 Dose estimates and verification

MCNP modelling generated a heat map of the dose rate across the irradiation facility (Figure 4.3). “Binned” dose rates were extracted at each of the treatment group locations for comparison to TLD data (Table 4.1).

Table 4.1 shows that under the ideal conditions of the MCNP modelling (*i.e.* that there was no shielding of any description in the facility and only the distance reduced the dose rates), the predicted dose rates were slightly higher than those measured by the EPDs. The EPDs were direct measurements of the radiation beam at the specific point of the dose rate treatment within the facility and there were plastic shelving units in place that will have provided a (limited) shielding effect on the radiation beam. Furthermore, the samples closer to the rear of the facility may have been subject to reductions in the radiation beam by the glass jars present at the higher dose rates.

Table 4.1 also shows that the TLD data, averaged over the TLDs placed at each distance from the source. Generally, these are lower than the MCNP modelled dose rates for similar reasons to the EPD data but were reasonably consistent with the measured EPD dose rates.

Table 4.2 shows the TLD measurements of the shielding effects of the glass jar containing media, where the average difference in dose rate between the front and the back of each jar was 31%. The MCNP modelled estimate of the reduction in the dose rate through a jar containing water is shown in Figure 4.4. The difference in the dose rate front to back of the jar with water was calculated to be 30%. When the base of the jar was considered the reduction in dose rate through the solid glass was calculated to be 60%.

Table 4.1: Distance from the source for each dose treatment and dose range estimates for each treatment group using MCNP modelling, EPD's and TLD data.

Distance from source (m)	Modelled dose rate from MCNP (µGy h^{-1})	Dose rate from EPD (µGy h^{-1})	Dose rate from TLDs (µGy h^{-1})
Control	1	0.45	0.3
9	14	5	3
7	29	15	Not measured
4	96	84	87
2.6	235	Not measured	224
2	369	350	Not measured

Table 4.2: Shielding effects of the jars.

Jar	Front from TLDs (mGy)	Back from TLDs (mGy)	%Δ in TLD measurements
1	3.6	2.5	30
2	5.3	3.5	35
3	5.0	4.6	8
4	5.6	3.8	32
5	4.1	2.8	31
6	4.1	2.8	32
7	4.2	3.3	21
8	5.2	3.7	30
9	5.2	3.1	41
10	5.2	2.9	45
11	5.4	4.3	21
12	6.0	3.9	36

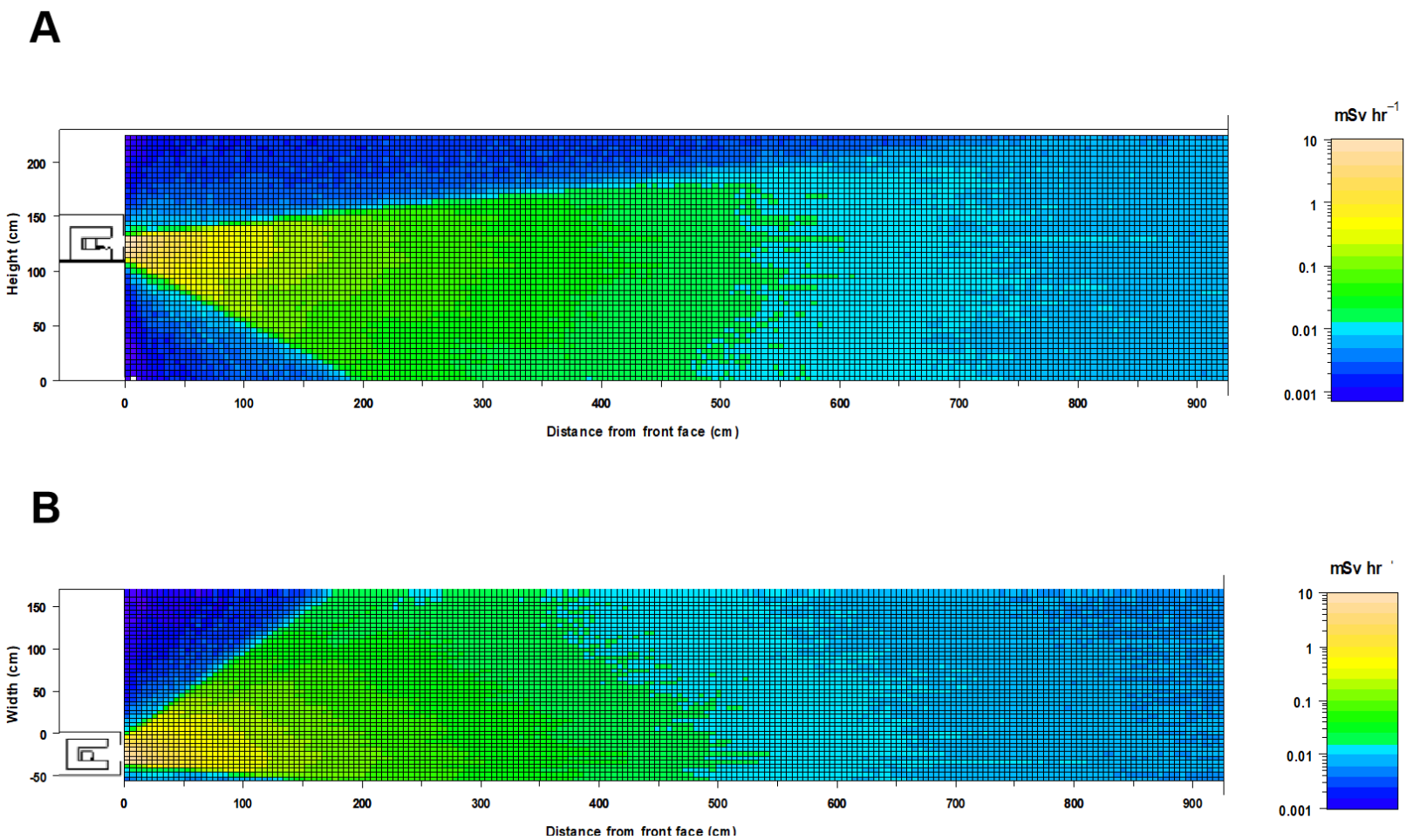
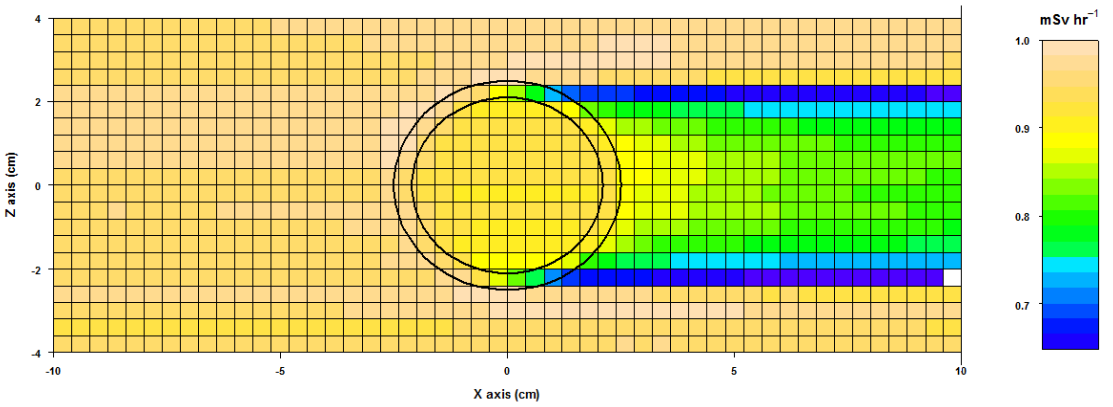


Figure 4.3: MCNP simulations output showing projected dose rates within the irradiation facility at the University of Stirling (A) side view of the radiation souce (B) birds eye view of the radiation source.

Figure 4.4: Figure showing the MCNP modelled dose rates at the front and back of the glass jar with A) representing a bird's eye view of the jar and B) a cross section through the jar showing the effect of the glass at the bottom of the jar.

A



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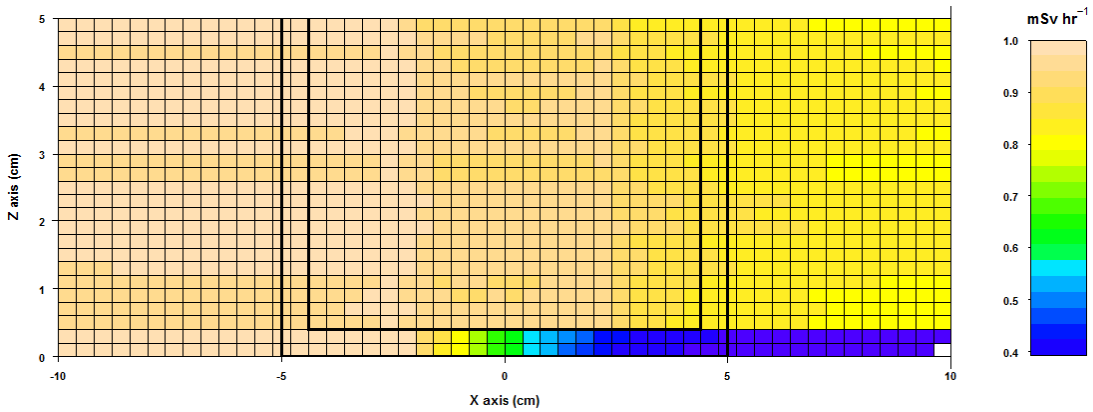

4.4.2 Dose rate significantly affects the total number of offspring produced

There was a significant quadratic effect of dose rate on the total number of offspring produced (GLM: χ^2_1= 61.08, p < 0.0001. Figure 4.5). This relationship was sustained after removing individuals that did not produce eight broods of offspring (GLM: χ^2_1= 20.22, p < 0.0001).

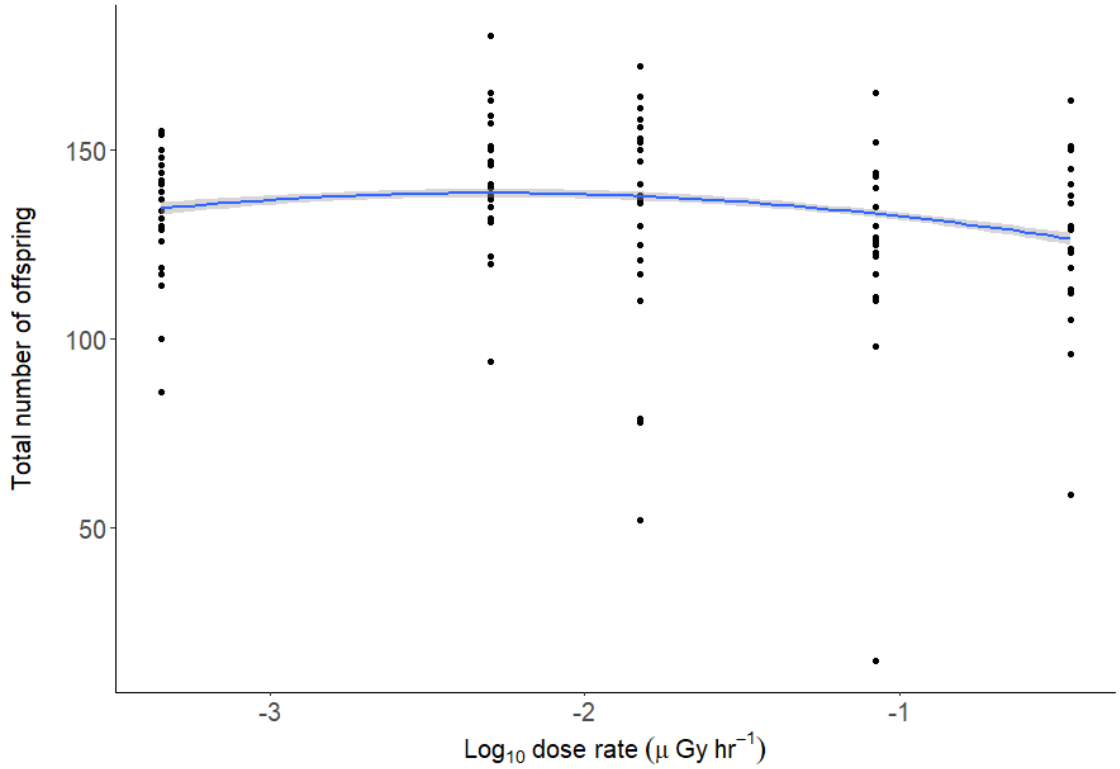

Figure 4.5: Relationship between the total number of offspring produced and Log_{10} dose rate. Points show the raw data. The line was fitted with a smoothing function in the *ggplot2* package, based on a generalised linear modelling approach with a Poisson distribution. The grey shading around the line denotes 95% confidence intervals.

4.4.3 No effect of dose rate on time until first brood release

The time taken until first brood release varied from experimental day 7 – 10. Dose rate did not have a significant impact on time taken until first brood release (CPH: coefficient =1.12 ± 0.78, p= 0.15. Figure 4.6).

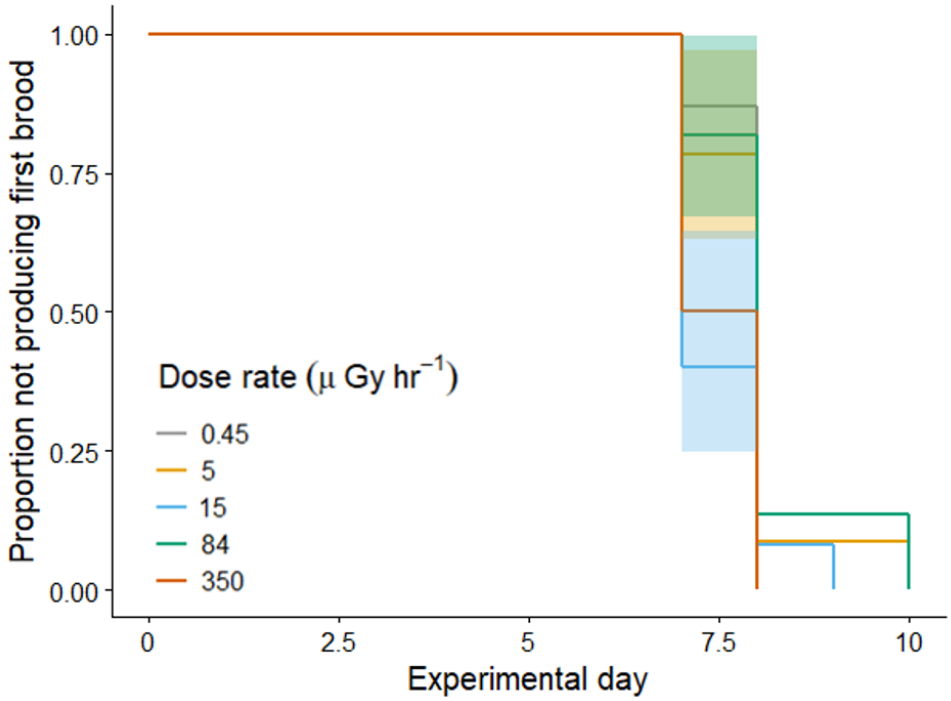

Figure 4.6: The effect of dose on the number of days until the first brood release.

4.4.4 No effect of dose rate on age-specific reproduction

There were no significant effects of dose rate (ANOVA: χ^2_1= 2.79, p= 0.1) or the interaction between dose rate and experimental day (ANOVA: χ^2_1= 0.03, p= 0.86) on age-specific reproduction (Figure 4.7).

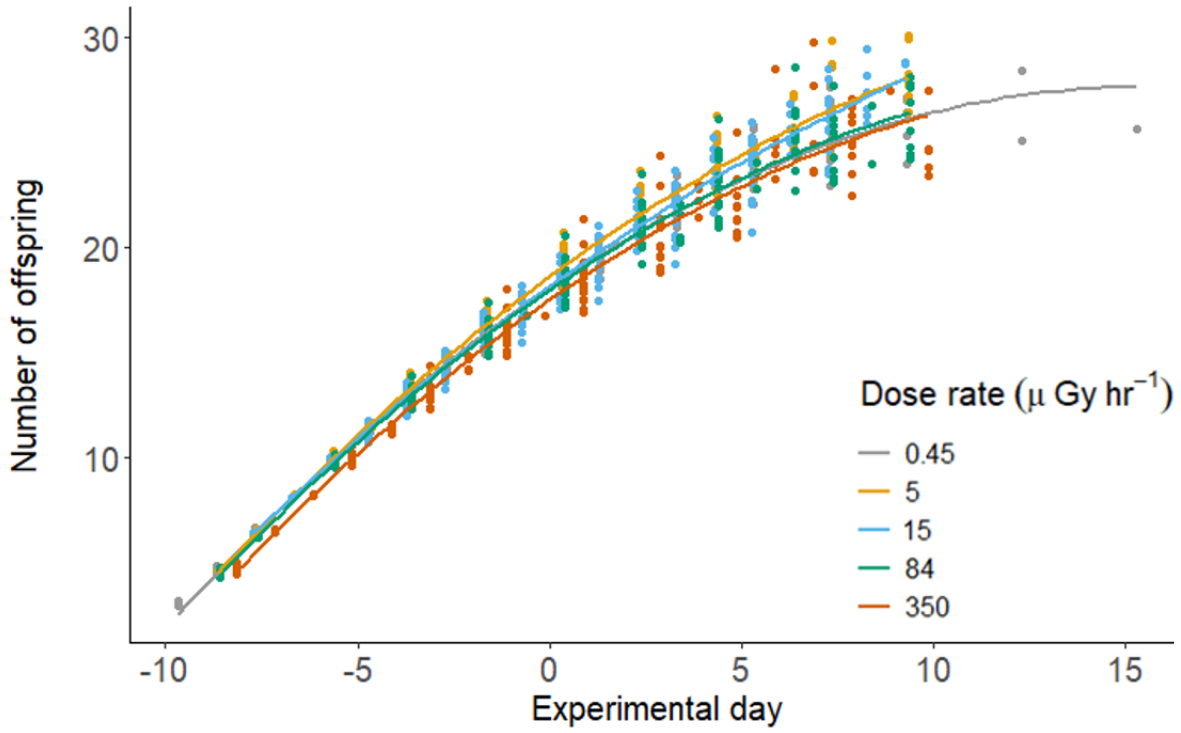

Figure 4.7: The effect of dose treatment on age-specific reproduction. Each line represents the reproductive effort predicted by the best-fitting generalised linear mixed effects model.

4.4.5 No effect of dose rate on survival

There was no effect of dose rate on *Daphnia* survival (CPH: coefficient = 0.07 ± 0.28, p = 0.81. Figure 4.8).

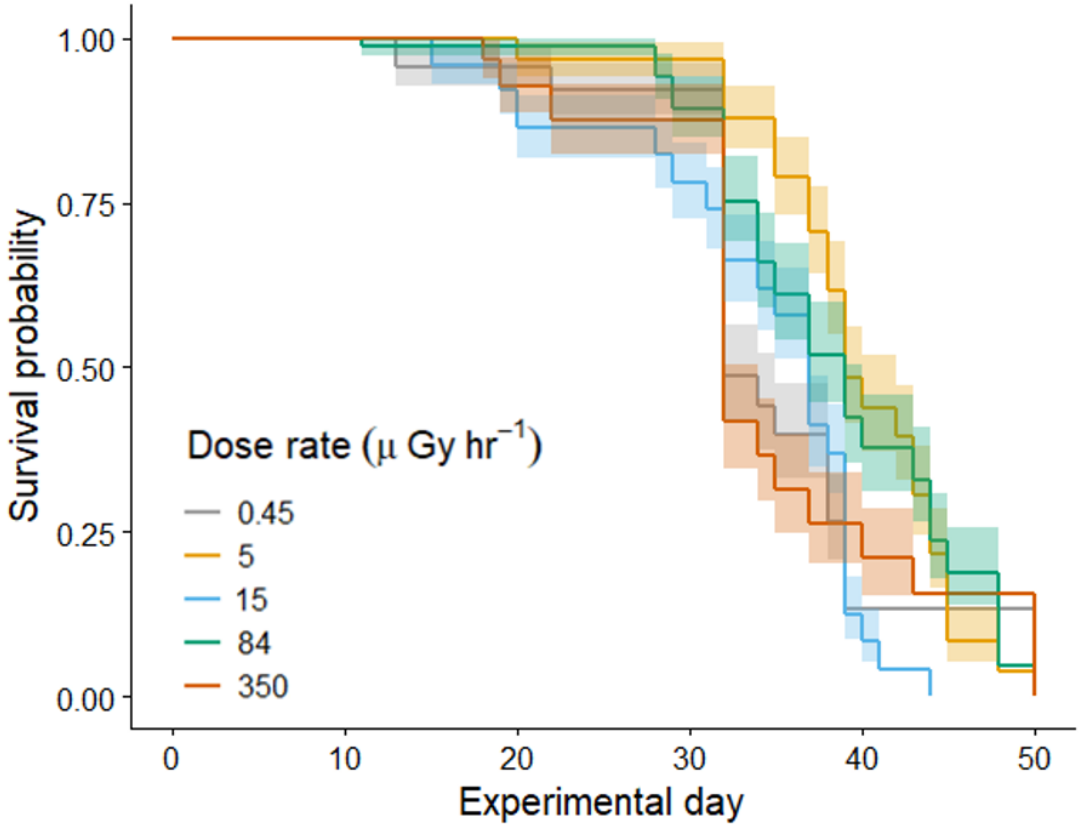

Figure 4.8: The effect of dose treatment on survival (survival probability ± 95% confidence intervals [CI's]). This graph shows the fixed effects only, calculated using Coxph models using the *survival* package (Therneau, 2015) and plotted using the *survminer* package (Kassambara *et al.*, 2018).

4.5 Discussion and conclusions

In this chapter, I used MCNP modelling to simulate the dose rates across the irradiation facility at the University of Stirling, which were verified using TLDs. The effect of radiation on *Daphnia* fitness was then tested, to investigate whether dose rates relevant to those in the CEZ are sufficient to cause sublethal effects on *Daphnia* fitness, and hence provide provisional data to inform the optimal experimental design for a transgenerational experiment in the following chapter. I found that the dose rates measured by the EPDs were consistent with those predicted by MCNP modelling and measured TLD data as shown in Table 4.1. Within a jar, the dose rate variation front to

back was in the order of 15% as determined by the MCNP model due to both distance through the jar and the media absorbing some of the radiation. *Daphnia* were not restricted in their movement within jar and were expected to move around within the radiation field. It is therefore not possible to measure the precise dose received by each individual *Daphnia*, however it is reasonable to assume that the dose rate averaged across the jar is a good approximation of that received by the *Daphnia*.

In the dose response experiment, there were significant effects of radiation on the total number of offspring produced, but on no other measures of fitness. Based on my current data and results shown in other experiments using *Daphnia* (Parisot *et al.*, 2015), it would be most appropriate to test transgenerational radiation effects using fewer dose treatments with increased replication, to maximise statistical power.

There was a significant quadratic effect of dose rate on the total number of offspring produced. One possible explanation is that low levels of ionising radiation cause shifts in life history strategy, due to anti-stress responses (as a result of oxidative stress from ionising radiation) (Dowling and Simmons, 2009; Monaghan *et al*., 2009). It has been suggested that increased reproductive investment results in an increased susceptibility to oxidative stress (Alonso-Alvarez *et al.*, 2004). These costs and exposure to higher dose rates could offer some explanation as to why a linear effect was not observed.

There were no effects of dose rate on the timing of reproduction. The day of first reproductive event varied between 7 and 10 days, which is unlikely to have provided sufficient exposure to generate a significant response, particularly as the experiment only tested within a single generation. Similarly, no significant effects were identified on age-specific fecundity. A transgenerational experiment conducted by Parisot *et al*. (2015) testing radiation exposures on *Daphnia* did not identify any reproductive delays in the fifth brood release from exposure to dose rates ranging from 70 to 3.5×10^4 µGy h^{-1} until the second generation. Therefore, any effects on reproductive timings within the dose rate range tested in the current chapter are likely to be very subtle in future generations. In order to provide enough statistical power to detect any effects on the timing of reproduction, it would be beneficial to use fewer dose treatments with increased replication when testing exposure across multiple generations.

Survival was also unaffected by dose rate. Other studies have not identified survival effects within single generation exposures at dose rates below 400 µGy h^{-1} (Gilbin *et al*., 2008; Parisot *et al.*, 2015), so it is unsurprising that no effect of dose rate was identified within the current Chapter. However, transgenerational experiments have identified effects from the second generation at dose rates from 4.7×10^3 µGy h^{-1}

(Sarapultseva and Gorski, 2013; Parisot *et al.*, 2015). To conduct an experiment across multiple generations, it is important that the majority of replicates survive until the final generation to minimise data loss. Based upon the findings here, and data from other transgenerational radiation experiments, I will test exposure across multiple generations at the highest dose rate (350 µGy h^{-1}) only, with the highest level of replication practically achievable (see Chapter 5). This will test the maximal number of replicates possible thus increasing statistical power for identifying any subtle radiation effects that might be observed.

The results from the current chapter provided data to inform the experimental setup in Chapter five, with which to test transgenerational radiation effects on *Daphnia* at dose rates relevant to those seen in Chernobyl. I observed radiation effects on total offspring, but not in age-specific fecundity. There were no radiation effects on survival. This suggests that any radiation effects at Chernobyl relevant dose rates are likely to be subtle and may not appear until later generations. The lack of survival effects suggests that the maximum achievable dose rate can be used, without excessive loss of *Daphnia* replicates in my transgenerational experiment.

Chapter five:

Contrasting effects of ionising radiation within and across generations

5.1 Abstract

Ionising radiation can affect both the supply of genetic variation by causing DNA mutations that are passed on to future generations in the germline and deplete variation by selecting against individuals that lack sufficient anti-stress mechanisms. Using multiple replicates of a single genotype of the facultatively sexual crustacean, *Daphnia pulex,* I dissect the effects of continuous exposure to ionising radiation on survival and fecundity across six asexual generations. I found that the effects of radiation exposure, in terms of reduced fecundity and shifts in reproduction, became apparent in the second generation of exposure, and that survival in radiation-exposed lineages declined over the generations. I also took offspring from radiation-exposed *Daphnia* and examined them in controlled conditions and uncovered different phenotypes to those *Daphnia* exhibited under continual exposure to radiation: continually-exposed animals shifted to early reproduction, consistent with a life history response to stress, whereas offspring from radiation-exposed mothers had delayed early reproduction, consistent with fitness decline due to mutation accumulation. Finally, I tested if exposure to radiation drove increased variation among radiation-exposed lineages across the generations. I found no evidence for this increased variance; I instead found that radiation-exposed lineages were more likely to go extinct before the sixth generation, and that these lineages exhibited a different schedule of reproduction prior to extinction. These findings suggest that exposure to ionising radiation can both induce a life history response to stress and increase the supply of mutations to populations, but that radiation-mediated selection acts as a check on any supply of genetic variation associated with radiation-induced mutation accumulation. Radiation-mediated selection could thus explain the lack of association between dose and life history variation among natural high radiation populations in Chapter two.

5.2 Introduction

Ionising radiation can potentially affect both the supply genetic variation to populations by causing mutation, and also deplete variation through selection. Radiation can cause mutations by damaging DNA directly; it can also cause mutations through indirect mechanisms such as the induction of reactive oxygen species (ROS) that subsequently cause DNA damage (Rhaese and Freese, 1968; Phillips *et al*., 1984; Halliwell and Aruoma, 1991; Riley, 1994; Box *et al*., 1995). Exposure to radiation also causes damage to other cellular machinery (cell membranes etc.) and can select against individuals that lack strong anti-stress mechanisms that decrease the number of damaging ROS molecules (Ramana *et al*., 1998; Khodarev *et al*., 2004; Diehn *et al*., 2009). There is also compelling evidence that both direct and indirect radiation-mediated effects can be transmitted to offspring and thus impact fitness-related traits such as reproduction, growth and survival of future generations (Dubrova *et al.*, 2000; Dubrova, 2003; Morgan, 2003a, 2003b; Zaka *et al*., 2004; Barber *et al.*, 2006; Buisset-Goussen *et al.*, 2014; Nohara *et al.*, 2014; Parisot *et al.*, 2015). So, in order to better understand how radiation exposure affects organismal, and ultimately population fitness, one must examine how both mutations and non-mutational effects (such as phenotypic plasticity or epigenetic effects) (Via and Lande, 1985; Ma *et al*., 2010; Antwih *et al*., 2013) can reach across generations to influence fitness-related traits.

Mutations nearly always lead to a loss of fitness (Timofeeff-Ressovsky, 1940; Muller, 1949, 1950). Muller's Ratchet hypothesis predicts that the inevitable accumulation of mutations in asexual lineages leads to an inexorable loss of fitness over generations, eventually leading to extinction (Muller, 1964). This is because it is not possible for an asexual individual to produce offspring with fewer deleterious alleles than itself. Indeed, mutation accumulation is hypothesised to be as a major reason for why sex (and not asexual reproduction) is the dominant mode of reproduction, despite it being hugely costly (Muller, 1964; Kondrashov, 1988; Charlesworth, 1990). Sex results in genetic recombination, which breaks apart gene complexes and can shuffle mutations, exposing them to selection (Haldane, 1937); sex therefore allows a mother to produce offspring that are free from her mutations (Kondrashov, 1988; Charlesworth, 1990). It is also important to note that mutations can arise both in germline and somatic cells (Sturtevant, 1937; Baer *et al*., 2007). In sexually reproducing organisms, germline mutations are passed on to offspring while somatic mutations are only expressed in the affected generation. In contrast, the lack of recombination means that asexually reproducing organisms pass on both somatic and germline mutations to their offspring (Muller, 1964; Crow and Kimura, 1965). Sex can also affect inheritance independent of

genetic recombination. Sex can reset the elaborate set of switches that turn genes on and off (the epigenome), and thus influence the expressed phenotypes of offspring, *i.e.*, transgenerational phenotypic plasticity (Shea *et al*., 2011; Rando and Chang, 2012; Verhoeven and Preite, 2014). So, in order to dissect the how mutations and non-mutational effects of radiation affect fitness, one must control for the effects of sex and recombination.

I overcame these issues using an organism that can reproduce both sexually and asexually and which naturally inhabits a region with high levels of ionising radiation: *Daphnia pulex*. I established a genetically homogeneous *Daphnia* isofemale line by asexually propagating a single animal sampled from near the Chernobyl Exclusion Zone (CEZ). I was therefore able to bypass the confounding effects of genetic recombination and epigenetic resetting (Muller, 1964; Lynch *et al*., 1993; Feng *et al*., 2010; Verhoeven and Preite, 2014) and quantify how exposure to ionising radiation shapes fitness across six (asexual) generations using a controlled laboratory experiment.

I hypothesised that there would be an overall decline in the fitness of *Daphnia* exposed to radiation over multiple generations, due to increased mutational load. Fitness was assessed as survival, age-specific reproduction and total offspring production. Declines in survival would be demonstrated by earlier death in the radiation treatment compared to the control treatment across generations (Figure 5.1A). Decreased fitness would be shown in total offspring production as reduced offspring output across generations, compared to the control treatment (Figure 5.1B). Reduced fitness in age-specific fecundity would be represented by delays in brood releases (Figure 5.1C and D).

I was further able to test the relative contributions of exposure in previous generations and within-generation continuous exposure on organism fitness, by comparing fitness in the offspring of radiation-exposed animals and control animals under background radiation dose rates. I predicted that the fitness of offspring of previously radiation-exposed lineages would decrease across generations compared to unexposed lineages, consistent with mutation accumulation. Offspring from previously exposed lineages may exhibit higher fitness than radiation treatment *Daphnia* due to lack of within-generation exposure (Figure 5.1).

Finally, I tested if there was divergence among lineages in radiation-exposed, but not control lineages. Mutation accumulation would be expected to generate more phenotypic variation in exposed lineages, whereas non-genetic fitness responses

would result in a decline in variation due to a similar phenotypic response. Increased divergence would be evident as increased variation in the responses of exposed lineages across generations, compared to unexposed lineages.

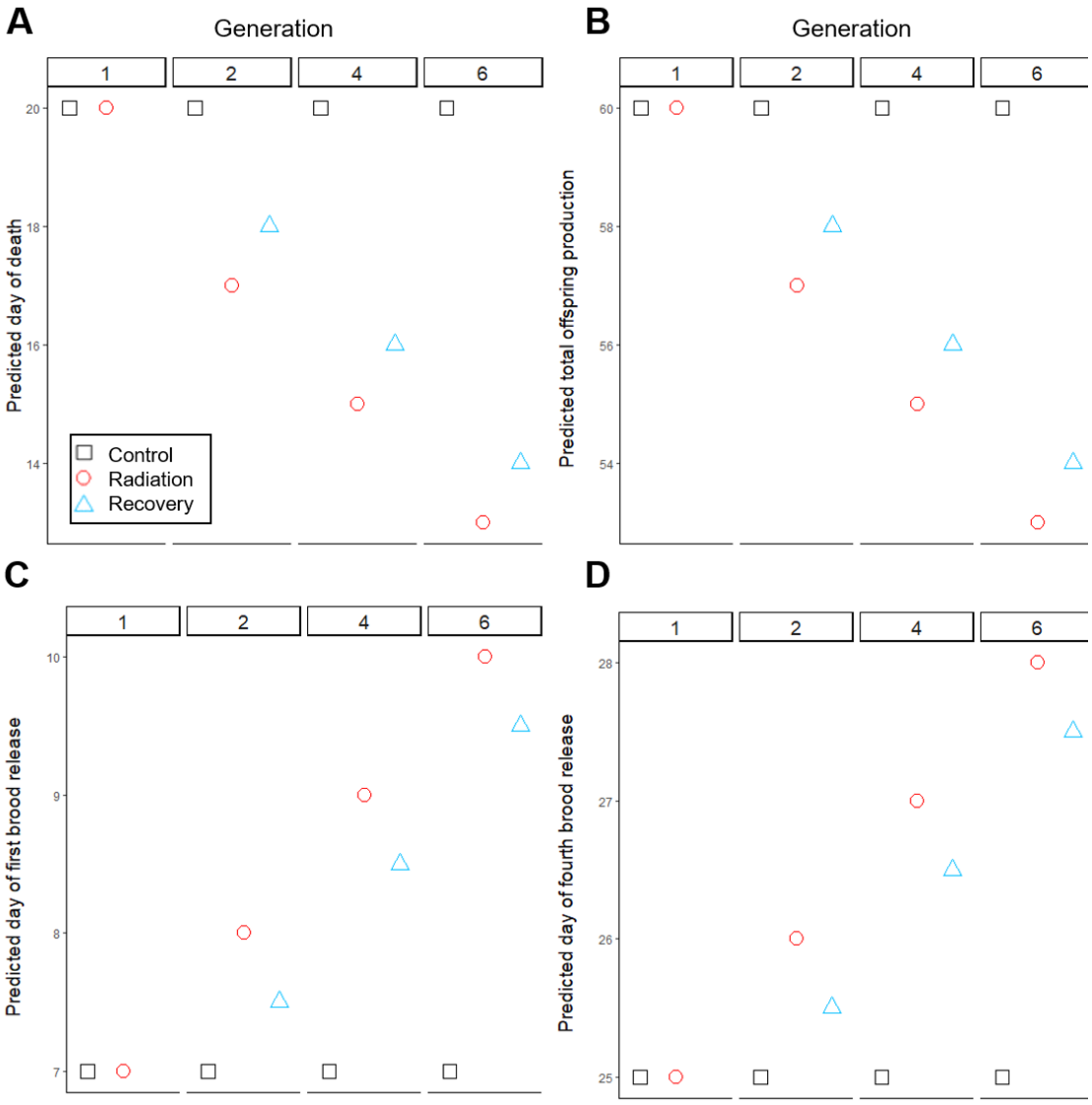

Figure 5.1: Predicted effects of treatment on *Daphnia* fitness. (A) Median day of death for *Daphnia* in control, radiation and recovery treatments in each generation. (B) Mean total number of offspring produced by each treatment group in each generation, error bars show the standard error values. (C) Median day of first brood release for *Daphnia* for each treatment group in each generation. (D) Median day of fourth brood release for *Daphnia* for each treatment group in each generation.

5.3 Materials and methods

5.3.1 Study organism

I used a single *Daphnia* genotype for the experiment. This was collected from Vediltsy Lake in Slavutych, situated near the Chernobyl Exclusion Zone (CEZ, see Chapter 2, section 3.2 for information on sampling site and protocol). This genotype was geographically relevant for Chernobyl populations, but had not experienced high level

exposures (see Chapter 2, Table 2.1). The sampled *Daphnia* genotype was maintained as an isofemale line in a 60 mL glass jar containing 50 mL *Daphnia* media (ADaM; see Kluttgen *et al*., 1994) and fed 1.0 ABS *Chlorella vulgaris* algal cells per day (where ABS is the optical absorbance 650 nm white light). In preparation for the experiment, 20 maternal lines were maintained for three generations to remove variation due to maternal effects. All *Daphnia* were maintained on a 12:12 hour light cycle at 25 °C in the irradiation facility at the University of Stirling.

5.3.2 Dosimetry

Daphnia were exposed to ionising radiation in the University of Stirling irradiation facility. The irradiation facility contains a ^{137}Cs source which provides dose rates in the range of approximately 5 – 3500 µGy h^{-1} over a nine metre distance (see Figure 4.2). The source is surrounded by lead shielding, arranged to emit radiation in a single direction across the experimental area. Each experimental unit consisted of an individual daphnid within a 60 mL glass jar containing 50 mL *Daphnia* media. Where generations were not yet established or had already died out, jars containing media, but no daphnid were maintained to ensure that the same dose rate was achieved in all jar positions throughout the experiment. Experimental units were setup in one of two treatment areas, arranged in rows; the control area, situated outside of the radiation beam, at a dose rate of 0.45 µGy h^{-1}, or the radiation treatment area, where the dose rate was 350 µGy h^{-1}. Mrem Electronic Personal Dosimeters (EPDs, Ludum Model 23) were used to verify that an even dose rate was received across each row. Due to shielding effects, the experimental units were systematically rotated within each treatment group daily throughout the experiment, to ensure an average even dose rate was received. The dose rates received by each daphnid were predicted using the Monte Carlo Code for Neutron and Photon Transport (Briesmeister, 1986; Brown, 2003). These were verified using thermoluminescent dosimeters (TLDs), including the front, back and within the jars to calculate the shielding effect of the glass and water as described in Chapter four.

5.3.3 Experimental setup

Six successive generations were maintained throughout the experiment, either in the control or in the radiation area. Generation one was established from the third brood release of a maternal line that was maintained under the environmental conditions described in section 5.3.1 for three generations. Fifty experimental units were set up in the control area of the facility (henceforth, control treatment) and fifty experimental units were setup in the radiation area (henceforth, radiation treatment).

As each replicate in generation one released its first brood, a single neonate was placed into a new jar to form an experimental unit in generation two within the same treatment area, which continued for successive generations until generation six (Figure 5.2). A new treatment group, termed the “recovery” treatment was introduced by removing neonates from the radiation treatment and maintaining them in the control area. One additional neonate was taken from the radiation treatment in generations one, three and five and placed into a new jar to create a new experimental unit for the recovery treatment for generations two, four and six (Figure 5.2). *Daphnia* were checked daily for reproduction and mortality over 30 days in generations one, two, four and six. Reproduction was measured for the first four brood releases per individual and recorded as the day of brood release and the number of offspring per brood. Mortality was recorded as the day of death for each individual daphnid.

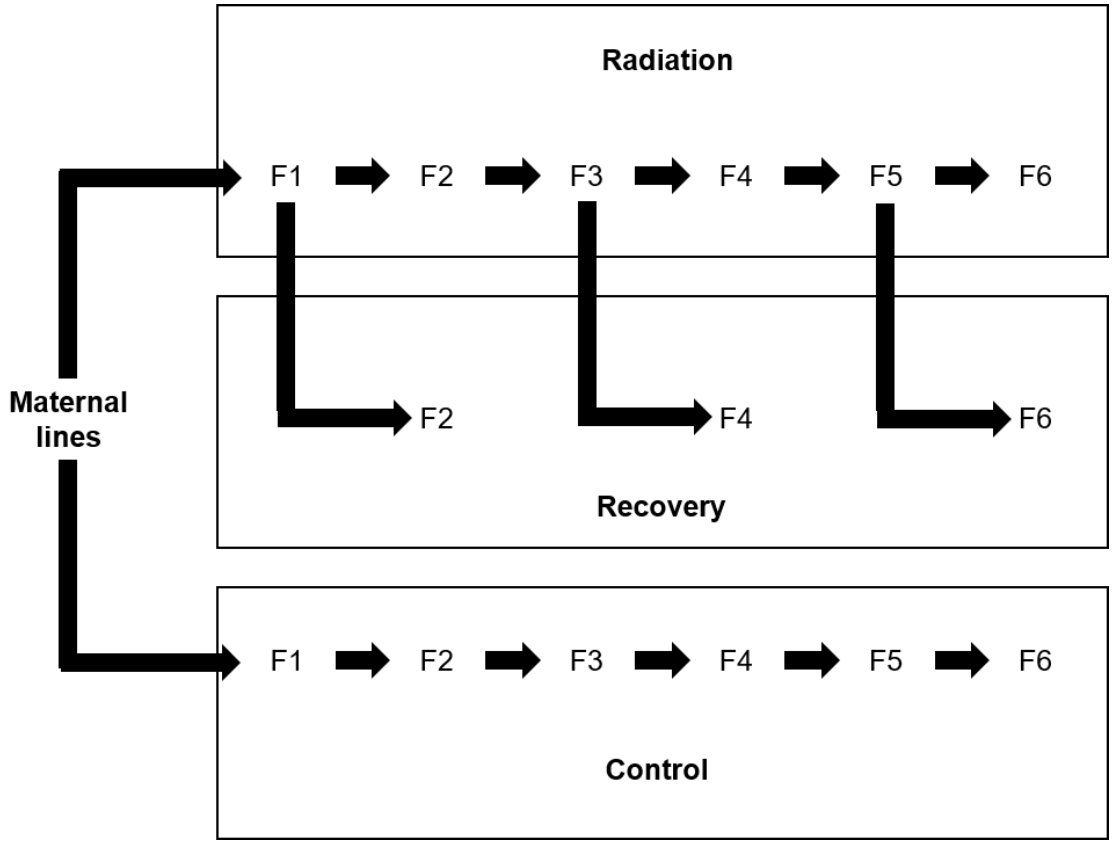

Figure 5.2: Experimental setup showing *Daphnia* taken from common maternal lines and setup in either the radiation or control treatment. Lines were maintained for six consecutive generations, labelled F1 – F6. Every second generation, *Daphnia* were removed from the radiation treatment and setup in the control area as a recovery treatment group.

5.3.4 Statistical analysis

Analysis was conducted using R version 3.4.3 (R Core Team, 2017). Data for the control treatment across generations was assessed for variation in survival, total offspring production, the day of first and fourth brood release and age-specific reproduction according to the methodology below. Due to variation in the control treatment across generations (see results in Appendix I), each generation was analysed separately.

First, I tested the hypothesis that *Daphnia* fitness would decline across generations in radiation-exposed, but not control treatments, consistent with accumulation of deleterious mutations. Specifically, I tested how treatment (control, radiation, recovery) affected *Daphnia* early survival using Cox's Proportional Hazard's (CPH) models (*survival* package; Therneau, 2015), with experimental day of death as the survival term. Next, analysis of how treatment affected age-specific reproduction was completed using linear mixed effects (LME) models for each generation (using *lme4* package; Bates *et al.*, 2015). These tested the number of offspring produced on each experimental day, with treatment group included as a fixed effect. The experimental days were mean-centred so that both the linear component (mean-centred day) and polynomial component (mean-centred day squared) of age-specific fecundity could be included in the model. A random regression term was included in the model, with experimental day fitted as the intercept for each replicate, to allow the slope to vary for each individual tested within the experiment. Type II sums of squares assessments were used to determine the significance of factors included in the model (using the *Anova* function within the car package) (Fox and Weisberg, 2019). *Post hoc* testing was performed using the Tukey test (*glht* function; Johnson and Omland, 2004).

In addition to the age-specific reproduction analysis, I also separately examined the timing and size of individual broods. Brood size analysis was completed using LME to test the number of offspring depending on each brood; fixed effects included treatment, generation and interactions between brood and treatment and between generation and treatment. A random regression term was included to allow the slopes to vary for each individual, where brood was fitted as the intercept. Significant parameters were identified using Type II sums of squares testing. To assess the timing of each brood release, I first tested the normality distributions using a Shapiro–Wilk test and then examined the effects of treatment on the timing of the first and fourth brood releases using CPH models, with the day of first and the day of fourth brood releases respectfully fitted as the survival terms. I then tested how total early offspring

production (the first four broods) was affected by radiation treatment. This was done using generalised linear models (GLMs) assessing total offspring production by treatment group with a Poisson error distribution.

Next, I tested my second hypothesis: that any radiation-mediated effects would disappear once lineages were transferred from radiation to control treatments (*i.e.,* when they were placed in the recovery treatment). The same analysis for key fitness parameters was used as described for the first hypothesis (survival, total offspring and age-specific reproduction). Where significant effects were identified, pairwise *post hoc* comparisons were conducted using Tukey tests to determine which dose treatments were different from each other (using the *multcomp* package; Hothorn *et al.*, 2017).

I tested whether variation in lineages increased across generations in the radiation treatment but not in the control. Again, I used LMEs (using the *lme4* package; Bates *et al.*, 2015). I tested between two model fits which both contained the number of offspring per brood as fixed effects and a random regression term using brood as the intercept for each individual replicate. The first model contained a regression term for lineage, with a treatment by generation interaction fitted as the intercept. Whereas the random regression term in the second model contained generation only as the intercept. An a*nova* was used to determine whether there was a significant difference between the models.

Finally, I examined whether lineages destined for extinction, *i.e.,* lineages that died out before generation six, had significantly different patterns of fecundity to those lineages that survived the duration of the experiment. I did this by fitting separate GLM models, with a Poisson distribution, testing whether the total number of offspring varied by treatment. I analysed generations one and four, with extinction status, treatment and their interaction included as fixed factors. I then analysed the effects of extinction on the timing of production of the first and fourth brood release. I did this by fitting separate CPH models to generation one and generation four data, with the respective day of first and fourth brood release as the survival term and extinction status, treatment and their interaction included as fixed factors.

5.4 Results

5.4.1 Radiation exposure reduces survival probability across generations

There was no effect of radiation exposure on *Daphnia* survival in generation one (CPH: $\chi^2_1 = 0.62$, $p = 0.43$. Figure 5.3A), where four out of fifty individuals died from each treatment group during the experiment. In generation two, there was an overall effect of

treatment on *Daphnia* survival (χ^2_2= 8.85, p< 0.05. Figure 5.3A), where control *Daphnia* had a higher survivorship than radiation-exposed *Daphnia*, (CPH: coefficient = 1.43 ± 0.56, z= 2.55, p < 0.05, see Appendix N), and recovery *Daphnia* (CPH: coefficient = 1.26 ± 0.58, z=2.16, p < 0.05); there was no difference in survivorship between recovery and radiation-exposed *Daphnia* (CPH: coefficient = 0.00 ± 0.39, z= -4.4, p = 0.66. Figure 5.3A). There was a total of four deaths in generation two for the radiation and recovery treatments and only one death in the control treatment during the experiment. Radiation and recovery treatments had a median lifespan of 16 and 17 days respectively, whereas the median lifespan of the control treatment was 23 days. Summary data for this Chapter are included in Appendices H1-H4 and survival plots for each generation are included in Appendix J.

Treatment also significantly affected survival in generation four (χ^2_2= 20.59, p< 0.0001. Figure 5.3A), where both the radiation and recovery treatment groups had a lower survivorship than in the control treatment (radiation verses control, CPH: coefficient = 1.81 ± 0.54, z= 3.35, p< 0.001. Recovery compared to control, CPH: coefficient = 1.85 ± 0.54, z= 3.43, p< 0.001). There were no significant differences between the radiation and recovery treatments in generation four (CPH: coefficient = 0.00 ± 0.29, z= 0.162, p= 0.87). Both the control and recovery treatments had a median lifespan of 29 days, whereas the radiation treatment had a median lifespan of 26 days. The longer lifespan in the control group shown in Figure 5.3A, is likely to be due to the low number of individuals that died during the experiment (n: control = 2, radiation = 6, recovery = 6).

Survival effects of treatment were also present in generation six (χ^2_2= 8.97, p< 0.05. Figure 5.3A). The control treatment also had a higher survival probability than in the radiation treatment (CPH: coefficient = 1.21 ± 0.56, z= 2.16, p < 0.05. Figure 5.3A). However, survivorship in the recovery treatment was significantly lower than the radiation treatment (CPH: coefficient = 0.0 ± 0.56, z=2.28, p < 0.05) but not the control (CPH: coefficient = -0.05 ± 0.71, z= -0.07, p = 0.95). Although the recovery treatment had a lower median lifespan of 19 days than in the radiation treatment with 22.5 (median lifespan in the control treatment was 34 days), only one individual died from both the recovery and control treatments, whereas four individuals died in the radiation treatment.

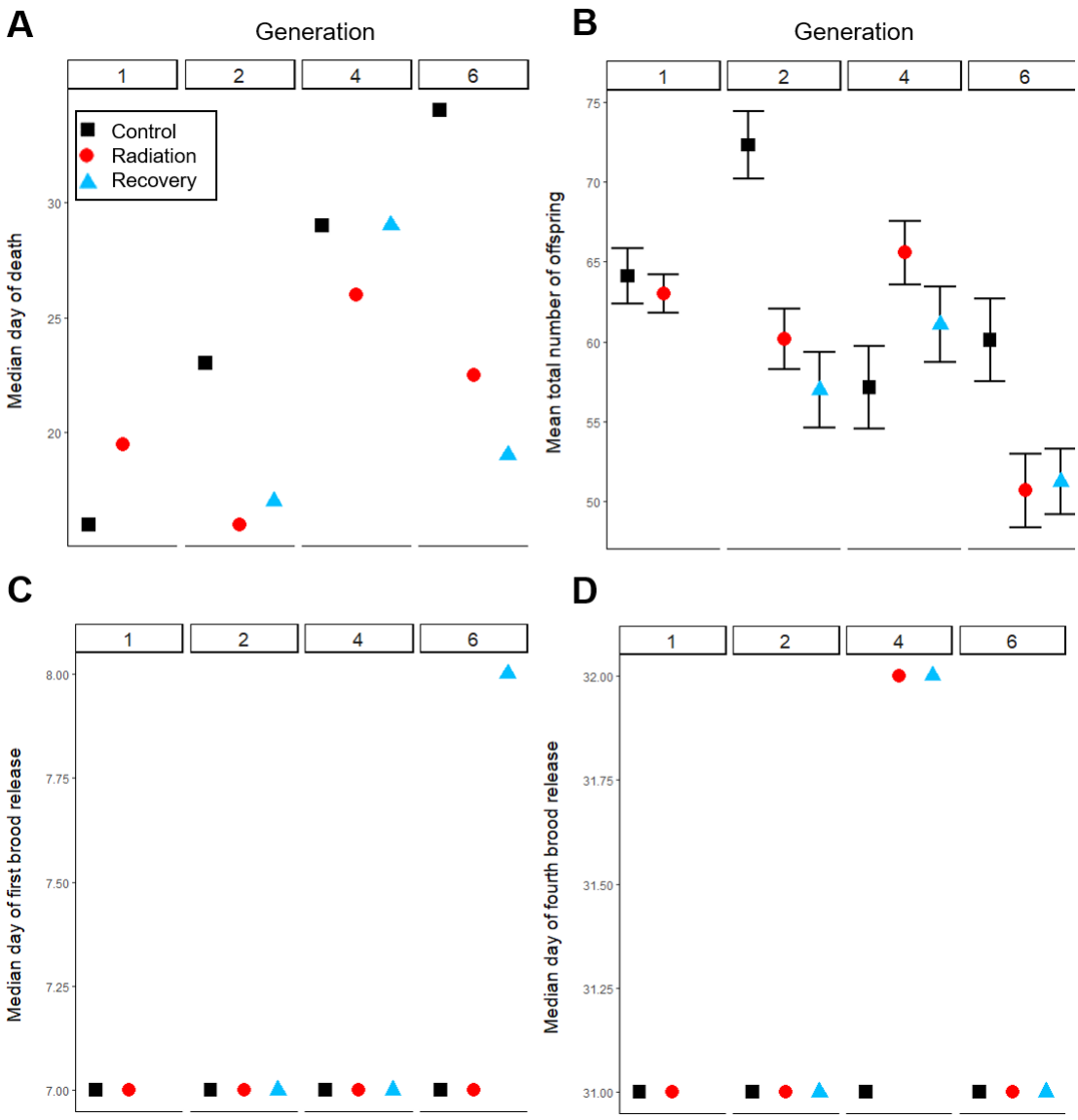

Figure 5.3: Summary statistics showing the effects of treatment on *Daphnia* fitness. (A) Median day of death for *Daphnia* in control, radiation and recovery treatments in each generation. (B) Mean total number of offspring produced by each treatment group in each generation, error bars show the standard error values. (C) Median day of first brood release for *Daphnia* for each treatment group in each generation. (D) Median day of fourth brood release for *Daphnia* for each treatment group in each generation.

5.4.2 Age specific reproduction 1: overall fecundity

There was no difference between control and radiation treatments in age specific reproduction in generation one (ANOVA: χ^2_1= 1.45, p= 0.23. Figure 5.4A). There was a significant effect of treatment in generation two (ANOVA: χ^2_2= 31.37, p < 0.0001.,

Figure 5.4B) where control *Daphnia* had consistently higher fecundity than both radiation (coefficient= -2.25 ± 0.47, z= -4.83, $p < 0.0001$) and recovery treatments (coefficient= -2.29 ± 0.48, z= -4.77, $p < 0.0001$) (see Figure 5.4B). Recovery and radiation treatments did not vary in the timing of reproduction from each other (coefficient= -0.04 ± 0.48, z= -0.09, p= 1.0) and both produced fewer offspring than the control. There was no variation in age-specific reproduction between treatment groups in generation four (ANOVA: $\chi^2_2= 0.6$, p= 0.75, Figure 5.4C). There was a significant effect of treatment in generation six (ANOVA: $\chi^2_2= 11.31$, $p < 0.01$, Table 5.1). However, post hoc testing revealed that treatment groups did not vary from each other. There was no significant treatment effect in generation six when examining control and radiation groups only (ANOVA: $\chi^2_1= 0.31$, p= 0.86. Figure 5.4D), so it is likely that this significance is due to delayed production of the fourth brood release in one of the replicates from the recovery treatment, as shown in Figure 5.4D. This is explored further in separate analysis for the timing of each brood release (section 5.4.3).

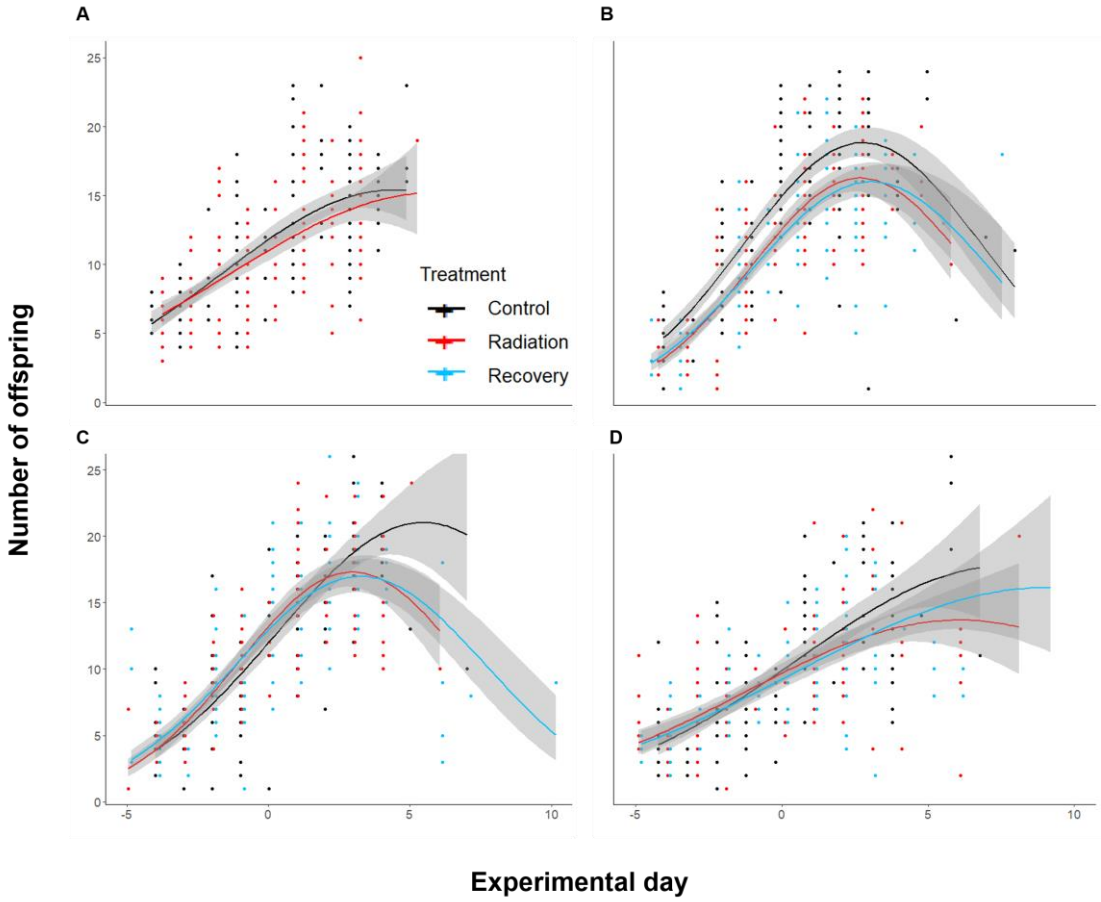

Figure 5.4: Age specific fecundity across five clutches for (A) generation one, (B) generation two (C) generation four and (D) generation six. Points show the raw data and lines were predicted using the GLM smoothing function with a Poisson distribution and show 95% confidence intervals.

5.4.3 Age specific reproduction 2: separate brood sizes and timings

Analysis of separate brood sizes and timing showed that brood size did not vary depending on treatment group (LME, control and radiation: estimate= 1.17 ± 0.66, z= 1.77, p= 0.18, LME, recovery and control: estimate= 0.83 ± 0.82, z= 1.01, p= 0.56. LME, recovery and radiation: estimate= -0.34 ± 0.94, z= -0.36, p=0.93).

There were no significant differences between radiation and control treatments in time until first brood release in generation one (χ^2_1= 1,15, p< 0.05. Figure 5.3C), where the median day of first brood release was day 7 in each treatment (The mean day of the first brood release was 6.8 ± 1.4 in the control treatment and 6.6 ± 1.2 in the radiation treatment.). However, the timing of the fourth brood release was significantly affected by treatment (χ^2_1= 10.38, p< 0.01. Figure 5.3D), where the radiation treatment had a slightly later day of fourth brood release (CPH: coefficient= 0.74 ± 0.23, z= 3.24, p < 0.01). The median day of the fourth brood release in both treatments was day 31. The mean day of the fourth brood release was 29.7 ± 1.4 in the control treatment and 29.7 ± 1.2 in the radiation treatment. Survival plots for the timing of the first and fourth brood releases for each generation are included in Appendices K and L respectively.

There were significant differences in time until first brood release in generation two (χ^2_2= 6.59, p< 0.05. Figure 5.3C), between the control and radiation treatments (CPH: coefficient= 0.52 ± 0.25, z= 2.34, p < 0.05) and the control and recovery treatments (CPH: coefficient = 0.5 ± 0.24, z= 2.06, p < 0.05), where the radiation and recovery treatments reproduced earlier than in the control treatment. However, there was no significant difference between the radiation and recovery treatment groups (CPH: coefficient = 0.0 ± 0.24, z= -1.1, p = 0.91. Mean day of first brood release, control = 7.7 ± 2.1, radiation = 7.1 ± 1.9, recovery 7.2 ± 2.0. Median day in all treatments was day 7).

The timing of the fourth brood release in the second generation was also significantly affected by treatment (χ^2_2= 12.76, p< 0.01. Figure 5.3D), where the radiation treatment was significantly different from both the recovery and control treatments (radiation versus control, CPH: coefficient= 0.86 ± 0.24, z= 3.63, p < 0.001. Radiation versus recovery, CPH: coefficient= 0.0 ± 0.24, z= -1.99, p < 0.05). There was no significant difference between the recovery and control treatments (CPH: coefficient = 0.37 ± 0.24, z= 1.53, p= 0.13). The median day of the fourth brood release for generation two was 31 in all treatments. On average, the radiation treatment produced the fourth brood of offspring the earliest (mean number of offspring in radiation = 29.8 ± 1.7, control = 30.64 ± 1.1, recovery = 30.3 ± 1.9), which is also evident in Figure 5.4B.

In generation four, treatment significantly affected age at first reproduction (χ^2_2= 10.64, p< 0.01. Figure 5.3C), with significant differences between control and radiation treatments (CPH: coefficient= 0.9 ± 0.27, z= 3.31, p < 0.001), where the control treatment had a later average day of first brood release (control: 7.6 ± 2.0, radiation: 6.7 ± 2). The recovery treatment was also significantly different from the radiation treatment (CPH: coefficient = 0.0 ± 0.27, z= -2.14, p < 0.05) but not the control (CPH: coefficient = 0.29 ± 0.27, z= 1.09, p = 0.28.). The mean day of first brood release was slightly later in the recovery treatment than in radiation (7.2 ± 2.3). The median day of first brood release in all treatments was day 7.

The timing of the fourth brood release in generation four was also significantly affected by treatment (χ^2_2= 17.22, p< 0.001. Figure 5.3D). The radiation treatment was significantly different from both the control and recovery treatments (radiation versus control, CPH: coefficient= 0.95 ± 0.27, z= 3.54, p < 0.001. Radiation versus recovery, CPH: coefficient= 0.0 ± 0.28, z= -3.93, p < 0.0001) and there was no difference between control and recovery treatments (CPH: coefficient = 0.17 ± 0.28, z= -0.6, p= 0.55). The radiation and recovery treatments had a later median day for the release of the fourth brood of offspring at 32 days, compared to 31 in the control treatment. However, the mean day for the fourth brood release in the radiation treatment was earlier than that of both the control and recovery (mean day of fourth brood release in radiation= 30.4 ± 2, control = 31.3 ± 2.0, recovery = 31.1 ± 2.3. Also see Figure 5.4C).

Age at first reproduction was also affected in generation six (χ^2_2= 5.7, p< 0.05. Figure 5.3C). There were no differences between control and radiation treatments in the timing of first brood release in generation six (CPH: coefficient= -0.05 ± 0.28, z= -0.17, p= 0.86). The recovery treatment was significantly different from both the control (CPH: coefficient = -0.63 ± 0.31, z= -2.03, p < 0.05) and radiation treatments (CPH: coefficient = 0.0 ± 0.3, z= -2.05, p < 0.05), with a later mean day of first brood release (recovery = 8.4 ± 2.1, radiation = 7.5 ± 2.3, control = 7.6 ± 2.6). The median day of first brood release in the recovery treatment was day 8 and day 7 for both control and radiation treatments. There were no significant effects of treatment on the release of the fourth brood (χ^2_2= 4.02, p= 0.13. Figure 5.3D) in generation six.

5.4.4 Radiation effects on total offspring production

There were no significant differences between radiation and control treatments in total offspring production in generation one (GLM: χ^2_1= 0.4, p= 0.53. Figure 5.3B). In generation two, the control treatment produced significantly more offspring than both the radiation and recovery treatments (control and radiation treatments, GLM: χ^2_1=

42.31, p < 0.0001. Figure 5.3B. Control and recovery treatments, GLM: Estimate = -0.23 ± 0.03, z= -8.0, p< 0.0001. Mean total number of offspring in control: 72.3 ± 2.1, radiation: 60.2 ± 1.9, recovery: 57 ± 2.4). There were no significant differences between the radiation and recovery treatments (GLM: Estimate = -0.06 ± 0.03, z= -1.7, p= 0.19). See Appendix M for plots of total offspring produced by each treatment in each generation.

In generation four the radiation treatment produced significantly more offspring than the control treatment (GLM: χ^2_1= 17.59, p < 0.0001. Mean radiation = 65.6 ± 2.0, mean control = 57.1 ± 2.6. Figure 5. 3B). There were no differences between recovery and control (GLM: Estimate = 0.07 ± 0.04, z=-1.93, p= 0.13) or recovery and radiation (GLM: Estimate = - 0.07 ± 0.03, z= -2.18, p= 0.08, mean total number of offspring in recovery = 61.1 ± 2.3).

In generation six, the radiation treatment produced significantly fewer offspring than the control treatment (GLM: χ^2_1= 20.71, p < 0.0001. Mean radiation = 50.7 ± 2.3, mean control = 60.1 ± 2.6. Figure 3B). The recovery treatment was significantly different from the control (GLM: Estimate = -0.16 ± 0.04, z= -4.1, p< 0.001) but not the radiation treatment (GLM: Estimate = 0.01 ± 0.04, z= 0.29, p= 0.96. Figure 5. 3B) where the control group produced the most offspring overall (mean total number of offspring in recovery = 51.3 ± 2.1). Variability in total offspring across generations is explained by the results shown in section 5.4.6.

5.4.5 No difference in lineage variation between treatment groups across generations

Variation in age-specific reproduction for each lineage across generations was assessed by examining the variance associated with the random effect containing lineage nested within either generation interacting with treatment or generation only. Including treatment interacting with generation did not improve the model fit (LME: χ^2_{15}= 9.9, p= 0.83).

5.4.6 Evidence for life history shifts in lineages that are destined for extinction

A total of 11 lineages in the radiation treatment went extinct by generation six and 15 in the control treatment (see Appendix O). Note that lineages can still be maintained if organisms die during the experiment provided they produced offspring before they died. It is also important to note that all recovery animals originate from radiation-exposed lineages. The total number of offspring produced was not affected by the extinction of *Daphnia* lines in generation one (GLM: χ^2_3= 48.85, p= 0.92. Figure 5.5A).

However, there was a significant effect in generation four (GLM: χ^2_4= 1792, p< 0.01), where *Daphnia* from lineages destined for extinction produced fewer total offspring than from non-extinct lineages (control extinct: 51.2 ± 4.3. Control non-extinct: 58.7 ± 3. Radiation extinct: 54 ± 10.0. Radiation non-extinct: 66.8 ± 1.9. Figure 5.5B).

In generation one, the day of the fourth brood release was not affected by the extinction of *Daphnia* lineages across treatments (CPH: χ^2_1= 3.14, p= 0.08. Figure 5.5C). Extinction did significantly affect the day of fourth brood production in generation four (CPH: χ^2_1= 5.35, p< 0.05). *Daphnia* from radiation-exposed lineages that went on to go extinct produced their fourth brood earlier than non-extinct lineages (non-extinct: 13.5 ± 0.2. Extinct: 12 ± 1. Figure 5.5D). Whereas control lineages destined for extinction produced their fourth brood later than non-extinct control lineages (non-extinct: 14.3 ± 0.1. Extinct: 15.2 ± 0.7).

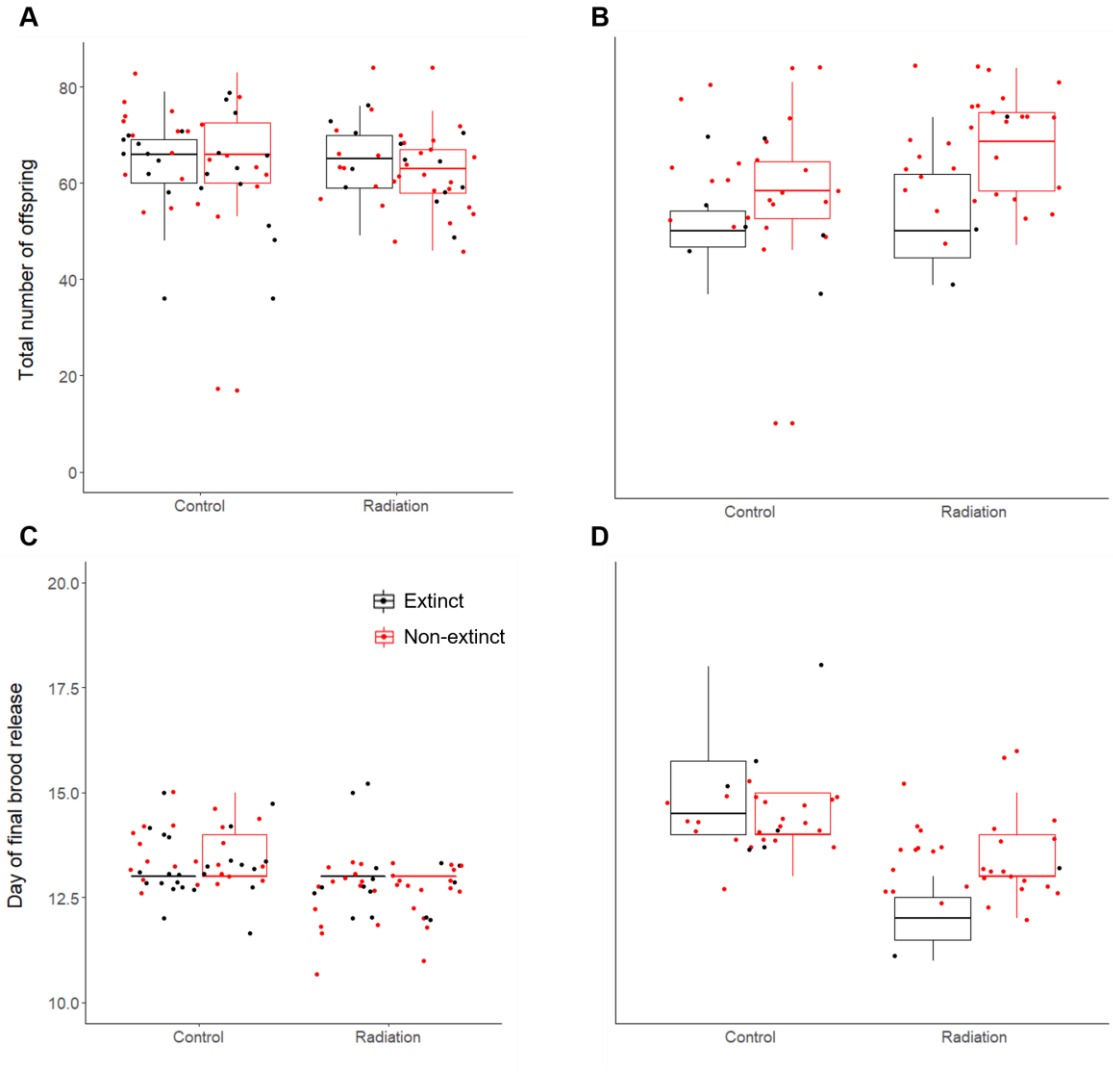

Figure 5.5: Patterns of reproduction on lineages that either survived or went extinct before generation six. (A) The total number of offspring produced in generation one, (B) the total number of offspring produced in generation four, (C) the day of the fourth brood release in generation one and (D) the day of the fourth brood release in generation four. The box shows the upper and lower quartiles within the data and the line within each box shows the median value. The lines outside of each box show the range of the data. The dots show the raw data values.

5.5 Discussion and conclusions

Radiation has long been known to negatively affect fitness in many organisms (*e.g.* Phillips & Coggle, 1988; Zaka *et al.*, 2004; Parisot *et al.*, 2015). *Daphnia* populations living in natural environments experiencing high levels of radionuclide contamination

(such as the CEZ) still experience much lower levels of exposure than those that are usually tested in the laboratory (> ~180 µGy h^{-1} in Chernobyl [Chapter two], to upwards of approximately 2 x 10^5 µGy h^{-1} in these laboratory studies: Marshall, 1962; Sarapultseva and Gorski, 2013; Sarapultseva and Dubrova, 2016). *Daphnia* living in high radiation field populations (*e.g.* in Chernobyl) also have the potential to transmit the effects of radiation exposure across generations, either as mutations or through non-mutational epigenetic/plastic effects (Morgan, 2003a, 2003b). It is therefore important that transgenerational assessments of radiation effects include dose rates and exposure scenarios that could realistically occur in the environment, for example after a major radiological incident such as Chernobyl. I conducted such an experiment using a *Daphnia* genotype sampled from Slavutych, a city located approximately 50 km from the CEZ (Intelligence Systems GEO, 2008). This genotype will be representative of a similar geographical location to Chernobyl, without exposure to high dose rates (Table 2.1). In this Chapter, *Daphnia* were exposed to three treatments: control (0.45 µGy hr^{-1}), radiation (350 µGy hr^{-1}; a Chernobyl-relevant dose) or recovery (where offspring from the radiation treatment were placed in control conditions, see Figure 5.2), and quantified fundamental fitness-related traits across *Daphnia* generations. I found clear evidence that radiation exposure led to reduced survival in later generations, consistent with the accumulation of deleterious mutations. I also found evidence that radiation drives life-history shifts towards earlier reproduction. Furthermore, lineages that produced fewer offspring overall, or on days that deviated from the average were selectively removed from the experiment. In the control treatment, lineages that reproduced later than average became extinct by generation six, whereas in the radiation treatment lineages that were destined to become extinct reproduced earlier.

Radiation-exposed *Daphnia* suffered reduced survival compared to *Daphnia* in the control treatment in generations two onwards (see Appendix N). This pattern of survival was also observed within the recovery treatment (except for generation six, where the generally low mortality meant that there was insufficient statistical power to test the hypothesis). Organism survival is dependent on complex mechanisms that regulate and maintain cell cycle and DNA damage repair that are governed by many genetic pathways (Elledge, 1996; Brown & Baltimore, 2000; de Klein *et al.*, 2000; Takai *et al.*, 2000; Zhou and Elledge, 2000). So, where mutational frequency is high (increased DNA alterations have been observed at dose rates as low as 7 µGy h^{-1} in *Daphnia* consistent with increased mutational frequency, Parisot *et al.*, 2015), there are numerous opportunities for disruption to such mechanisms, which could eventually lead

to organism fatality. This is consistent with the idea that mutation accumulation in asexual lineages will lead to eventual extinction (Muller, 1964; Lynch and Gabriel, 1990; Charlesworth, Morgan and Charlesworth, 1993) and that DNA damage from ionising radiation will accelerate the process.

Whilst it was difficult to determine a clear effect of transgenerational radiation exposure on overall age-specific reproduction, analysis of the timing of the first brood release revealed an increasing delay across generations within the recovery treatment group. This is consistent with mutation accumulation in the germ line. Indeed, there is abundant evidence that oxidative stress (an effect of ionising radiation) selects for a shift in life resource allocation to somatic maintenance and increased longevity, at the expense of reproductive investment in early life (Alonso-Alvarez *et al*., 2004, 2006; Dowling & Simmons, 2009; Monaghan *et al*., 2009). It is, however, important to note that there was no evidence of an increasing delay in first reproduction in radiation-exposed *Daphnia* (though there was a delay in the production of the fourth brood in generations two and four). A possible explanation for these differences between radiation and recovery treatments is that the build-up of germline mutations across generations and the stress responses and somatic mutational damage associated with contemporary exposure to radiation have antagonistic effects which result in life history trade-offs. Indeed, it has been argued that physiological stress responses to environmental change can potentially limit effects on life histories and evolutionary responses (Ricklefs and Wikelski, 2002).

There were no clear directional effects of radiation on total offspring production in the radiation or recovery treatment groups across generations. Careful consideration needs to be taken in dissecting any subtle variation in total offspring between treatments. If physiological changes associated with continuous mutational input and complex life-history trade-offs are having opposing effects as previously discussed, this could explain the absence of a clear effect. However, the questions addressed in the current Chapter did not generate appropriate data to support this with certainty.

I also tested whether variation among lineages for age-specific reproduction increased across generations in radiation-exposed but not control lineages, as would be expected if mutations were causing increased genetic variation over time. I found no such effect. This led me to consider the possibility that mutationally compromised individuals in the radiation treatments either died before producing the subsequent generation (causing the extinction of inferior lineages from the experiment) or exhibited treatment-mediated life history changes that changes that differed between treatment groups. I found

compelling support for the second hypothesis. There was no evidence for radiation-induced extinction, but lineages destined for extinction were quicker to produce their fourth brood in generation four (but not generation one). *Daphnia* in the radiation treatment were continuously exposed and it is hypothesised that this led to an elevated level of somatic mutations, and that early reproduction in these lineages could be a resulting stress response to this situation. Other stresses are known to cause a shift to early reproduction in *Daphnia* (*e.g.,* infection with parasites: Chadwick and Little, 2005), and, whilst adaptive, such responses do not necessarily protect lineages from extinction (Hutchings *et al.*, 2012). The findings of this Chapter demonstrate selective disappearance as seen in other studies (Vaupel, Manton and Stallard, 1979; van de Pol and Verhulst, 2006), and are also consistent with the idea that the build-up of mutations causes an inexorable loss of fitness over generations, leading to extinction (Timofeeff-Ressovsky, 1940; Muller, 1949, 1950).

In summary, this Chapter examined the transgenerational effects of ionising radiation at dose rates equivalent to those seen in the CEZ, and the experimental design allowed me to dissect the effects of contemporary exposure to radiation from radiation exposure in previous generations. Using asexually reproducing *Daphnia* as a model organism, it was possible to bypass epigenetic resetting and the eradication of mutations through genetic recombination. I found evidence inferring mutation accumulation was occurring through my survival data and fecundity data. I also found evidence of phenotypic change consistent with the effects of continuous mutational input; these changes could plausibly counteract any life history trade-offs associated with parental exposure. Further analysis examining lineage extinction revealed that the selective disappearance of lineages that deviated from optimal reproduction, explaining why radiation-exposed lineages did not diverge over time. This selective disappearance is a prime explanation for the absence of radiation-driven variation observed in life history traits among contemporary populations in Chapter two. Future work should focus on molecular analysis of organisms exposed across generations, to further test the mutation accumulation hypothesis. In addition, further phenotypic work should be conducted to confirm whether continuous radiation exposure can limit life history responses.

Chapter six:

General discussion

In this thesis, I have combined laboratory and field approaches to test the effects of current dose rates across the Chernobyl Exclusion Zone (CEZ) on wildlife, using *Daphnia* as a model system. My first chapter outlined some of the current challenges in radioecology that have led to increased uncertainty with regards to whether current dose rates have a negative impact on wildlife (Beresford and Copplestone, 2011; Wickliffe and Baker, 2011; Garnier-Laplace *et al.*, 2013). I highlighted that some of the disagreements have been attributed to dosimetry approaches and insufficient consideration of confounding factors (Beresford *et al.*, 2008, 2012; Beresford and Copplestone, 2011; Garnier-Laplace *et al.*, 2013). I argued that a promising approach to address these uncertainties is to combine laboratory and field experiments, to generate an accurate dose response curve and ensure the effects of ionising radiation are understood both in isolation, and with respect to additional stressors.

Subsequently, Chapters two and three assessed the current state of Chernobyl *Daphnia* populations at both the phenotypic and molecular level respectively. Chapters four and five then focused on exposing *Daphnia* to dose rates relevant to those currently in the CEZ under controlled laboratory conditions, to determine the effects of ionising radiation on *Daphnia* lineages across multiple generations.

6.1 Effects of selection across Chernobyl populations

The data analysis for each chapter was conducted with the general hypothesis that *Daphnia* fitness would decrease with increasing dose rate. However, I found that radiation impacts were far more complicated than originally anticipated, due to different selective processes across levels of biological organisation. Initial examination of *Daphnia* sampled from across the CEZ in Chapter two showed that population fitness was not affected by dose rate and that other population-level factors had more of an influence on *Daphnia* fitness than radiation itself. Chapter three then confirmed that selection on *Daphnia* populations living within the CEZ was driven by factors other than dose rate. This was evidenced through inbreeding patterns that were independent of dose rate and significant correlations between genetic distance and Euclidian geographic distance. This suggested that geographical similarities and gene flow between nearby populations explained more genetic variation than dose rate.

Chapter three also showed that, whilst selection was governed by ecological factors independent of radiation, radiation did still contribute to the supply of mutations, evidenced by increasing genetic diversity with dose rate. Continuous exposure to ionising radiation has been shown to result in increasing mutational load over time (Breimer, 1988; von Sonntag, 2007). In sexually reproducing populations, genetic

recombination purges the genome of some of these mutations (Kondrashov, 1988; Charlesworth, 1990). However, *Daphnia* reproduce on a cyclically parthenogenetic basis, where asexual reproduction takes place throughout the spring/summer (Berg, 1931). Based on the time of sampling (7^{th} – 16^{th} July 2016), it is likely that the majority of *Daphnia* were reproducing asexually, meaning that both somatic and germline mutations should have been accumulating across lineages (Sturtevant, 1937; Muller, 1964; Crow & Kimura, 1965; Baer *et al.*, 2007, Chapter five). *Daphnia* sampled at this time are therefore likely to have accumulated a substantial mutational load. This is supported by the evidence for survival declines at 350 µGy h^{-1} (representative of high dose rates in the CEZ) over just six generations in Chapter five, consistent with mutation accumulation

Mutations can affect a wide variety of genetic pathways that impact on cell cycle maintenance and DNA damage repair mechanisms, leading to reduced organism survival (Elledge, 1996; Brown & Baltimore, 2000; de Klein *et al*., 2000; Takai *et al*., 2000; Zhou and Elledge, 2000). High mutation rates, including those driven by ionising radiation, are therefore likely to result in a shorter lifespan (as shown in Chapter five). However, early-life organism fitness is more valuable to a population than fitness in later life, because individuals are more likely to have reproduced in early life, supplying their genetic material to the population gene pool (Birch, 1948). Where multiple ecological pressures are acting on populations across the CEZ, selection is more likely to be driven by factors that directly influence early-life fitness (such as predation, food availability or sterility-inducing parasites, Lehmann, 1993; Anholt *et al*., 1998; Oro *et al.*, 2003; Creel & Christianson, 2008; Auld *et al.*, 2013). This may offer some explanation with regards to why mutational input from ionising radiation is not the primary selective force observed in the sampled *Daphnia* populations, despite clearly providing some mutational input (Chapter three).

6.2 Radiation-mediated selection in individuals

Selection becomes more complicated at higher levels of biological organisation, due to confounding ecological factors and evolutionary pressures within populations (Kauffman, 1993; Brèchignac and Doi, 2009). I therefore explored radiation effects in isolation in Chapters four and five to understand radiation induced effects that were not confounded by other ecological pressures. I conducted a pilot experiment in Chapter four to justify the experimental design for Chapter five. Chapter four showed that radiation effects at dose rates relevant to those across the CEZ were subtle, with a key observation for informing the experimental design for Chapter five being that survival

was unaffected. This meant that *Daphnia* could be exposed across multiple generations without losing a high number of lineages during the experiment. The subtle nature of radiation effects on *Daphnia* also meant that a high number of replicates was required at fewer treatments (*i.e.* different dose rates) to increase statistical power in examining radiation effects in Chapter five.

Chapter four demonstrated an overall decline in total offspring production within a single generation of exposure, which may seem inconsistent with Chapter five as this effect was not observed within the first generation of exposure. However, a simple explanation of this result is due to the fact that I counted the first eight brood releases for each individual in Chapter four but only the first four brood releases in Chapter five. *Daphnia* would have accumulated different doses due to differences in the duration of exposure in each experiment. Indeed, a reduction in total offspring in the radiation exposed treatment in Chapter five is seen in the second generation of exposure, consistent with the results in Chapter four.

Other laboratory-based studies have tested radiation effects across generations in *Daphnia* (Alonzo *et al.*, 2008; Parisot *et al.*, 2015; Trijau *et al.*, 2018), however, each of the studies only assessed radiation effects up until the third generation. Whilst this is standard practice in transgenerational assessment (Szyf, 2015), I recognised that effects at environmentally relevant dose rates (in the same order of magnitude as those in the CEZ) were likely to be subtle, such as delays in brood releases (Parisot *et al.*, 2015). My pilot experiment in Chapter four provided support for this and therefore prompted the experimental design in Chapter five, consisting of six generations and testing only two dose rates (350 µGy h^{-1} and 0.45 µGy h^{-1} as the control) to allow for 50 replicates within each treatment group. This allowed me to identify selective disappearance of inferior lineages which would have not been detected in a three-generation experiment.

It is important to note that although the radiation treatment clearly affected reproduction (exposed *Daphnia* that died produced fewer offspring overall and released their final clutch earlier than exposed *Daphnia* that survived), *Daphnia* lineages in the control treatment were also selectively removed from the experiment (specifically, those that produced their final clutches later than those that survived). This suggests that this phenomenon was not specific to ionising radiation. This could however, help to explain why no variation in reproductive measurements was observed in the wild populations in Chapter two.

6.3 Important considerations for testing between the laboratory and the field

One important consideration for laboratory studies to support field data, is to design experiments that reflect the types of exposures experienced by natural populations, as implemented in this thesis. Conducting studies that explore relevant dose rates to the same organisms in both the laboratory and the field allowed for realistic comparison between individual and population level effects. There are considerable data gaps for laboratory studies that investigate radiation-mediated effects at dose rates that represent environmentally relevant levels (Beresford. *et al.*, 2004; Garnier-Laplace *et al.*, 2004; Salbu, 2009). There is some evidence that extrapolation of effects to relevant dose rates does not provide an accurate representation of these effects due to the subtle nature of some of these changes (Brown, 1977; Tubina *et al.*, 2009). By conducting laboratory studies at appropriate dose rates, using *Daphnia* sampled from control regions of the CEZ, I can be confident in making comparisons between my laboratory and fieldwork.

Furthermore, by establishing a dose-response curve for the same organism in both the laboratory and the field (Garnier-Laplace *et al.*, 2013), it is possible to explore different hypotheses for where discrepancies may have arisen. My research benefits from having both phenotypic and molecular data to explore evolutionary effects in wild populations, which can help to explain the differences from my laboratory data. Combining laboratory and field data would also be beneficial in identifying a situation where other stressors act synergistically or antagonistically with ionising radiation, resulting in an increased or reduced effect on wildlife (Holmstrup *et al*. 2010; Coors and De Meester 2008).

My research provides further considerations for evolutionary processes that arise at the population level. My results are consistent with mutation accumulation in the laboratory (Chapter five) and the field (Chapter three). However, whilst negative fitness impacts were found in the laboratory, field organisms were unaffected due to population-level processes. This highlights the importance of varying evolutionary pressures across levels of biological organisation, as there are other agents of selection in ecologically complex communities (Kauffman, 1993; Brèchignac and Doi, 2009). In Chapter two, I discuss the relevance of my research to Polikarpov's proposed concept of organism, population and ecosystem responses to ionising radiation (Polikarpov, 1998). The radiation effects shown in individuals in Chapters four and five provide further support for this concept, because the detectable effects are eliminated at the population level. My work also provides further support in favour of the current perspective that

radioecology needs to consider ecological networks and population processes when estimating radiation effects on wildlife (Bréchignac, 2003; Mothersill *et al.*, 2019).

One key consideration for other work focusing on alternative species is that due to the cyclically parthenogenetic reproductive patterns in *Daphnia* (Hebert, 1987), I had the benefit of preserving the exact genotypes found in the field by allowing lineages to propagate asexually following sample collection. The majority of study systems do not have this benefit, meaning that sexual recombination could remove some of the radiation-mediated changes following collection (Haldane, 1937; Kondrashov, 1988; Charlesworth, 1990). This is something that could be explored further using the *Daphnia* model (see section 6.5).

It is also crucial to consider additional factors that affect population dynamics, for example population size. In Chapter three I identified that 92 out of 93 sampled individuals were multi-locus genotypes (MLGs) which indicated that populations were behaving as if they were purely sexual; it is therefore likely that *Daphnia* effective population sizes are very large (Allen and Lynch, 2012). Small populations are more likely to suffer following increased periods of stress that result in high mortality (such as high dose rates, Allendorf, 1983; Ellstrand & Elam, 1993). The large populations in the CEZ may have allowed *Daphnia* to cope with reduced survival resulting from ionising radiation (Chapter five). Particularly from higher past dose rates at the time of the accident, which were shown to cause reduced survival in a number of other species (Sokolov *et al.*, 1993; Hinton *et al.*, 2007; Geras'kin *et al*., 2008). This would be important to consider if replicate studies in highly contaminated landscapes sampled organisms from smaller lake populations.

In the laboratory, I investigated mutation accumulation in asexual lineages that were continuously exposed to the same amount of radiation over the duration of each experiment. Natural populations have the opportunity to move freely across a heterogeneous environment (particularly in terrestrial populations) (Mccarthy & Zachara, 1989; Thiessen *et al.*, 1999). It is important to consider that moving to a low radiation area, where there will be a reduction in mutational frequency, will provide a greater respite from any radiation-mediated selection and opportunity for successful DNA damage repair (Iliakis *et al.*, 2003) and thus potential removal of radiation effects.

6.4 Other anthropogenic stressors/major ecological changes

Daphnia living within the CEZ have experienced a complex exposure history, with high acute exposures following the accident in 1986, transport of radionuclides through the environment, decay of short-lived radionuclides and long-term chronic exposures

(Hinton *et al.*, 2007). However, this does not nullify the fact that research on organisms living within the CEZ can inform other researchers on the long-term effects of population responses to abrupt environmental change and chronic exposure to pollutants. My thesis offers useful insight to each of these situations: Chapters two and three assess the state of current populations over 30 years following the accident, and Chapters four and five dissect the contribution of current Chernobyl dose rates to effects on fitness. Collectively, Chapters two and three both show that whilst genetic diversity increased with dose rate, indicative of radiation-induced mutations, other ecological factors had greater selective impact on *Daphnia* populations than radiation itself. *Daphnia* are useful indicators of the state of aquatic ecosystems as they are primary consumers in food web dynamics (Miner, De Meester, *et al.*, 2012). The fact that over 30 years following the Chernobyl accident, *Daphnia* populations are not detrimentally affected by ionising radiation demonstrates the potential for recovery in aquatic ecosystems from rapid environmental change. This is further supported by other research on aquatic invertebrates across the CEZ showing no detrimental radiation effects on populations (Fuller *et al.*, 2017, 2018).

By testing the impacts of dose rates relevant to the highest dose rates currently within the CEZ on *Daphnia* in the laboratory in Chapters four and five, I begin to dissect the relevant contributions of current dose rates on individuals to the combined effects of current dose rates and long-term responses to rapid environmental change seen in Chapters two and three. I find that dose rates within the high end of the current range within the CEZ do impact on *Daphnia* fitness at the individual level and the effects on survival in Chapter five are consistent with the theory of mutation accumulation. This suggests that current dose rates could be maintaining the increasing diversity seen in Chapter three.

6.5 Scope for further research

Whilst the research presented in this thesis provides useful insight into *Daphnia* responses to long-term radiation exposure and can offer some insight into work on other anthropogenic stressors (Sections 1.10 and 6.4), it has also highlighted potential new areas of research. For example, Chapters two and three demonstrated that ecological pressures other than ionising radiation drove variation in phenotypic and molecular data. In an alternative ecosystem experiencing different selection pressures alongside high dose rates, there is the potential for stressors that act synergistically with ionising radiation to play the dominant role in shaping populations. There is conflicting evidence in the literature on the synergistic and antagonistic effects of other

stressors in combination with ionising radiation (Vanhoudt *et al.*, 2012). Therefore, it is important to test the effects of ionising radiation alongside other stressors that are likely to be present in natural environments.

Chapter five demonstrated that mutations accumulate across lineages, negatively affecting survival and that *Daphnia* lineages with inferior reproductive outputs were selectively eliminated. Natural *Daphnia* populations switch to the sexual component of their reproductive cycle when conditions become unfavourable, so it would be interesting to explore the effects of sexual selection following exposure to ionising radiation. Furthermore, the hatching success of ephippia has been shown to be affected by other environmental contaminants (Navis *et al.*, 2013; Möst *et al.*, 2015; Rogalski, 2015), highlighting the potential to examine radiation effects on hatching rates.

Chapter five used a single genotype to explore the opposing processes of mutation accumulation and selection. A similar experimental design could also be used to test the hypothesis that *Daphnia* from lake populations experiencing different dose rates are locally adapted to exhibit optimum fitness. This would involve exposing genotypes from different lake populations to the range of dose rates found across the CEZ.

6.6 Overall Conclusion

This thesis provides a detailed investigation on the effects of Chernobyl-relevant dose rates in both the field and the laboratory using *Daphnia* as a model system. I examined population-level fitness and the underlying population genetics in *Daphnia* representative of their respective lake populations collected from areas affected by Chernobyl derived radioactive fallout. I examined lakes that represented a radiation gradient in the CEZ and used two external controls for comparison. I found that although genetic diversity increased with dose rate, consistent with an increasing rate of genetic mutations, signatures of selection and population structure showed that alternative ecological factors were more dominant in shaping lake population fitness than radiation itself. In the laboratory, I found that constant exposure to ionising radiation across generations caused a reduction in *Daphnia* survival rate, also consistent with theory of increased mutation accumulation. However, close examination of reproductive fitness showed that inferior lineages were selectively removed across generations, stripping any reproductive variation. This could explain why no differences in fecundity were observed in field populations. Collectively, my work has demonstrated that ionising radiation does negatively impact individual *Daphnia* lineages at dose rates relevant to highly contaminated areas in the CEZ (350

µGy h^{-1} in the laboratory, ~ 180 µGy h^{-1} was the highest estimated field dose rate to *Daphnia*). However, any negative effects of radiation-mediated mutational input at the population level is eliminated by selection from other ecological sources.

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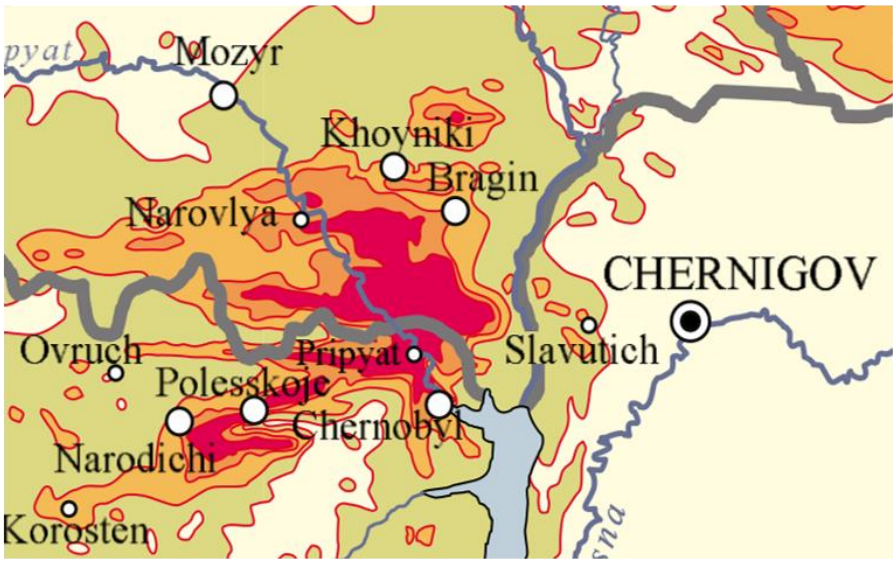
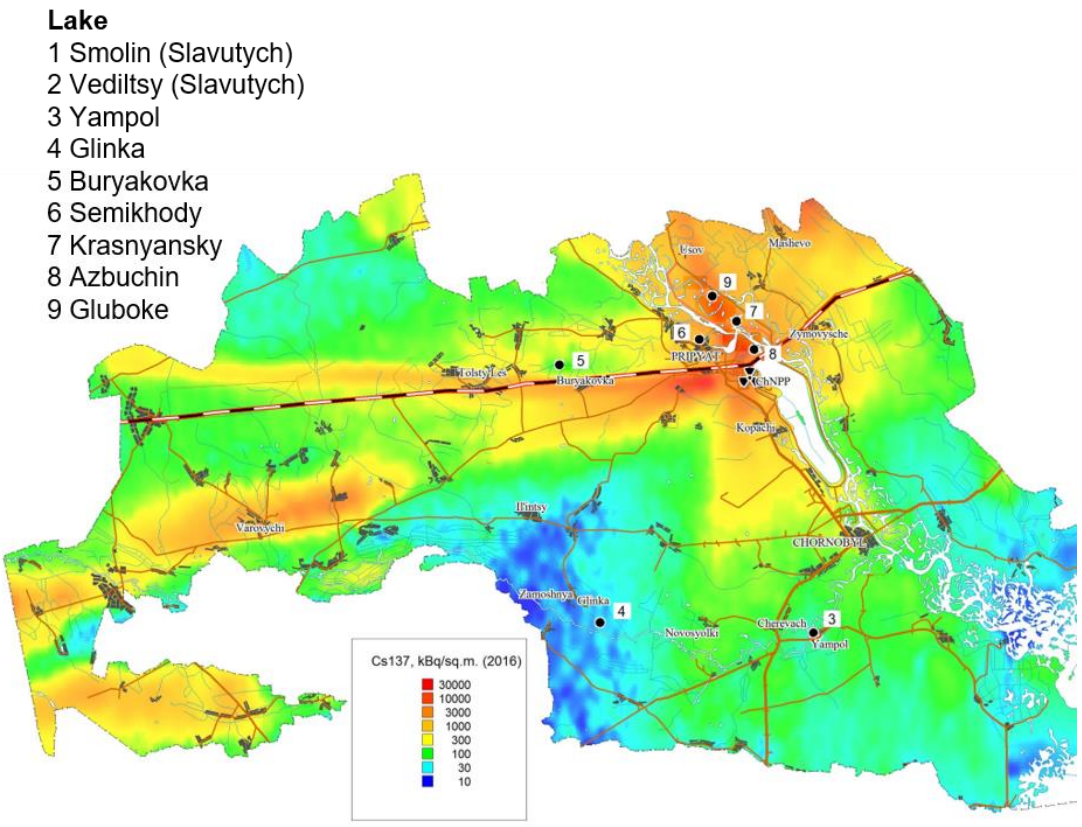
Appendices

A



B



Appendix A: (A) map showing the location of Slavutych (where sites 1 and 2 are located) relative to the Chernobyl Exclusion Zone (CEZ). Adapted from (UNSCEAR, 2000). (B) *Daphnia* sampling sites across the CEZ.

Appendix B: Summary data for Chapter two. *n* = the number of genotypes tested in the experiment once non-reproducing individuals were removed, DOD = day of death, MNO = mean number of offspring, B = brood, the range is given in days.

Lake	*n*	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3
Vediltsy	4	51	37 - 62	8	3 - 18	10	4 - 22	13	4 - 30
Yampol	2	51	45 - 67	8	4 - 14	12	6 - 20	19	9 - 33
Glinka	4	48	32 - 71	7	4 - 12	13	5 - 21	19	4 - 27
Buryakovka	4	45	24 - 61	7	4 - 13	11	5 - 17	19	9 - 33
Semikhody	4	57	29 - 69	8	2 - 14	13	4 - 25	13	6 - 27
Krasnyansky	6	53	29 - 67	8	2 - 19	11	4 - 29	15	4 - 35
Azbuchin	4	50	26 - 64	6	1 - 12	12	4 - 23	18	4 - 31
Gluboke	2	47	28 - 62	7	4 - 10	10	4 - 19	15	4 - 19

Lake	MNO B4	Range B4	MNO B5	Range B5	MNO B6	Range B6	MNO B7	Range B7
Vediltsy	18	4 - 42	21	5 - 36	21	4 - 46	22	6 - 46
Yampol	26	8 - 46	33	18 - 47	35	20 - 52	32	19 - 54
Glinka	27	14 - 41	31	14 - 45	32	10 - 48	26	6 - 49
Buryakovka	23	4 - 38	27	5 - 46	30	8 - 53	27	5 - 55
Semikhody	12	4 - 18	14	6 - 24	13	4 - 24	16	7 - 33
Krasnyansky	19	6 - 39	19	4 - 41	22	4 - 55	23	5 - 49
Azbuchin	25	10 - 41	29	4 - 46	31	6 - 58	28	7 - 46
Gluboke	15	5 - 27	18	6 - 31	23	9 - 34	21	9 - 25

Lake	MNO B8	Range B8	MNO B9	Range B9	MNO B10	Range B10	MNO B11	Range B11
Vediltsy	23	6 - 46	20	7 - 50	21	6 - 45	15	5 - 35
Yampol	31	13 - 52	23	9 - 42	21	6 - 41	23	10 - 49
Glinka	21	8 - 45	22	5 - 39	18	4 - 33	18	5 - 32
Buryakovka	21	6 - 59	23	8 - 49	21	5 - 44	22	10 - 55
Semikhody	17	7 - 31	22	5 - 38	20	5 - 37	19	4 - 48
Krasnyansky	22	5 - 57	21	4 - 49	21	4 - 35	18	5 - 34
Azbuchin	23	5 - 46	16	4 - 40	20	5 - 42	13	4 - 24
Gluboke	16	6 - 25	11	5 - 19	11	5 - 17	11	5 - 19

Lake	MNO B12	Range B12	MNO B13	Range B13	MNO B14	Range B14	MNO B15	Range B15
Vediltsy	19	4 - 42	15	10 - 21	11	4 - 17		
Yampol	26	4 - 52	21	8 - 40	15	15 - 15		
Glinka	13	4 - 27	17	10 - 33	13	13 - 13	20	20 - 20
Buryakovka	17	6 - 49						
Semikhody	17	8 - 32	15	6 - 28	18	14 - 21		
Krasnyansky	19	4 - 43	19	8 - 35	22	10 - 34		
Azbuchin	15	3 - 26	15	5 - 31	17	10 - 24		
Gluboke	11	7 - 15	10	10 - 10				

Lake	MNO B16	Range B16
Vediltsy		
Yampol		
Glinka	7	7 - 7
Buryakovka		
Semikhody		
Krasnyansky		
Azbuchin		
Gluboke		

Appendix C: ^{137}Cs, ^{90}Sr and ^{241}Am activity concentrations in water and sediment samples collected from Smolin lake in Slavutych. The activity concentrations for ^{239}Pu are not provided as the levels were too low to measure. Water (w) concentrations are in Bq l^{-1} and sediment (s) concentrations in Bq g^{-1} (dry weight).

^{137}Cs (w)	**^{137}Cs (s)**	**^{90}Sr (w)**	**^{90}Sr (s)**	**^{241}Am (w)**	**^{241}Am (s)**
0.03	0.13-0.59	0.03			0.002.0-0.015

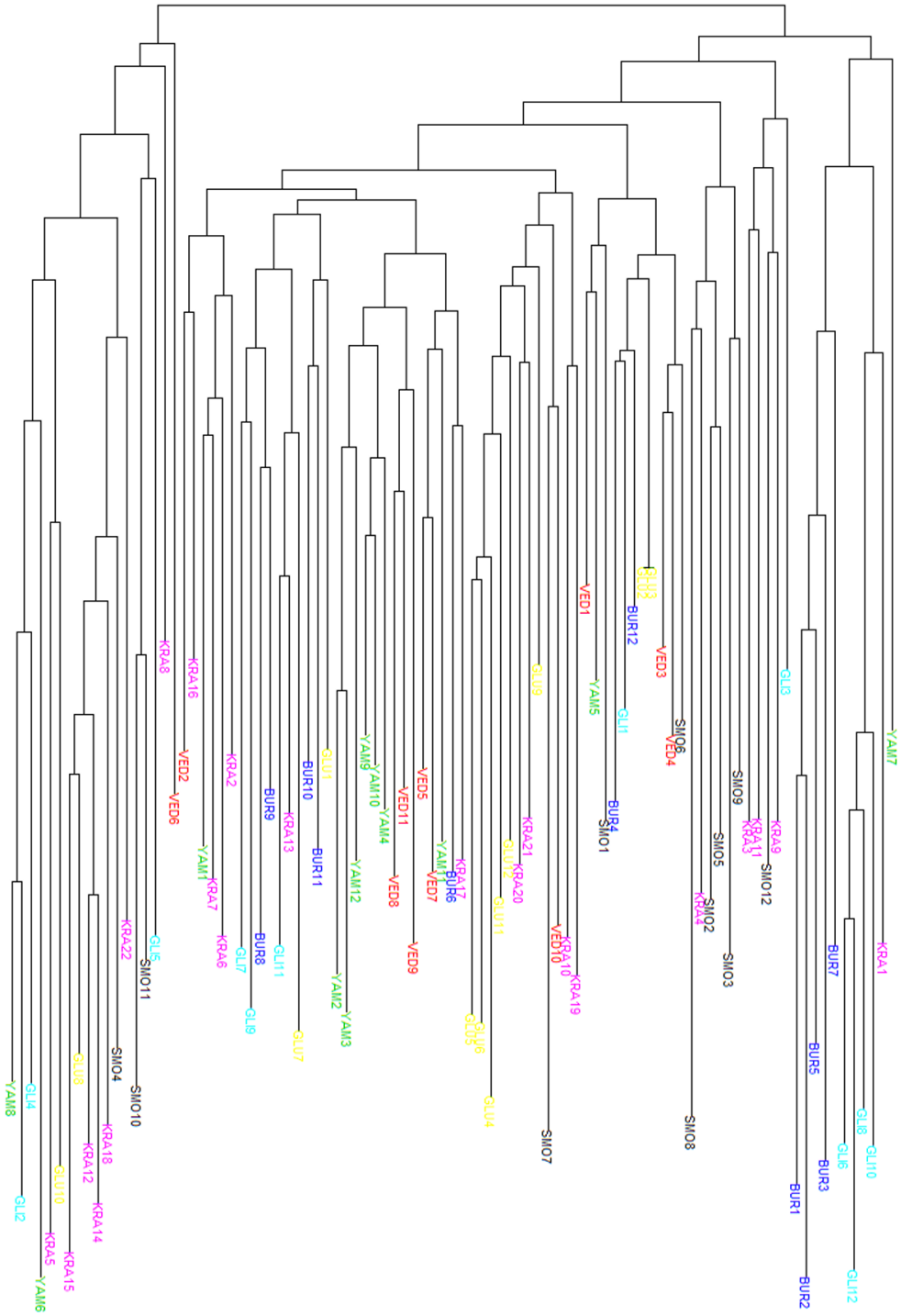
Appendix D: Hardy-Weinberg test for each locus. Significant values are highlighted in **bold**.

Locus	χ^2	d.f*	Pr(chi^2 >)	p-value exact
B064	76.61	6	**< 0.0001**	**< 0.0001**
B050	139.73	6	**< 0.0001**	**< 0.0001**
B008	84.22	6	**< 0.0001**	**< 0.0001**
B030	252.93	21	**< 0.0001**	**< 0.0001**
B031	164.07	21	**< 0.0001**	**< 0.0001**
B065	323.3	36	**< 0.0001**	**< 0.0001**
B135	10.04	1	**< 0.01**	**< 0.0001**
B088	11.45	6	0.08	0.06
B174	160.35	10	**< 0.0001**	**< 0.0001**
B075	222.68	28	**< 0.0001**	**< 0.0001**
B155	86.39	6	**< 0.0001**	**< 0.0001**

*d.f = degrees of freedom

Appendix E: Relative lake distances from Gluboke (the lake with the highest level of contamination, estimated ~ 180 µGy h^{-1}). Approximate distances are given in km.

Lake	Upper dose estimate	Distance
Smolin	0.12	70
Vediltsy	0.10	54
Yampol	0.20	27
Glinka	0.17	27
Buryakovka	1.77	13
Krasnyansky	55.79	3
Gluboke	181.15	0



SMO = Smolin, VED = Vediltsy, YAM = Yampol, GLI = Glinka,
BUR = Buryakovka, KRA = Krasnyansky, GLU = Gluboke

Appendix F: Genetic distance tree generated based on Nei's genetic distances (Nei, 1972), generated using the *ape* package in R (Paradis and Schliep, 2018).

Appendix G: Summary data for Chapter four. DOD = day of death, MNO = mean number of offspring, B = brood, the range is given in days.

Dose rate (µGy h^{-1})	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3
0.45	35	13 – 50	7	4 – 9	10	4 – 18	12	7 – 19
5	40	20 – 50	8	2 – 17	10	6 – 14	14	2 – 25
15	34	15 – 44	7	3 – 16	11	3 – 20	15	3 – 24
84	39	11 – 50	6	3 – 10	8	3 – 14	12	2 – 17
350	35	18 – 50	7	4 – 9	11	4 – 17	11	6 – 16

Dose rate (µGy h^{-1})	MNO B4	Range B4	MNO B5	Range B5	MNO B6	Range B6	MNO B7	Range B7
0.45	15	8 – 20	20	11 – 26	26	13 – 34	24	15 – 40
5	18	12 – 27	21	12 – 31	26	16 – 36	26	19 – 36
15	18	2 – 27	17	2 – 29	21	5 – 32	26	18 – 33
84	15	8 – 21	18	4 – 26	22	15 – 33	25	14 – 36
350	15	6 – 25	20	14 – 28	22	13 – 30	27	17 – 49

Dose rate (µGy h^{-1})	MNO B8	Range B8
0.45	22	15 – 30
5	26	17 – 37
15	27	14 – 39
84	25	16 – 35
350	23	13 – 40

Appendix H1: Summary data for experimental generation one in Chapter five. *n* = number of individuals in the experiment, DOD = day of death (if individuals died during the 30 experimental period), MNO = mean number of offspring, B = brood, the range is given in days.

Treatment	*n*	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3	MNO B4	Range B4
Control	46	15	10 - 21	7	4 - 10	10	4 - 18	14	6 - 23	15	7 - 23
Radiation	46	21	17 - 26	7	3 - 12	10	4 - 17	13	6 - 21	14	5 - 25

Appendix H2: Summary data for experimental generation two in Chapter five. *n* = number of individuals in the experiment, DOD = day of death (if individuals died during the 30 day experimental period), MNO = mean number of offspring, B = brood, the range is given in days.

Treatment	*n*	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3	MNO B4	Range B4
Control	45	23	23 - 23	6	1 - 16	11	1 - 23	17	6 - 23	19	11 - 28
Radiation	42	18	13 - 26	4	1 - 12	11	5 - 22	15	10 - 21	16	10 - 22
Recovery	46	18	10 - 20	5	1 - 16	11	4 - 18	15	5 - 22	15	7 - 20

Appendix H3: Summary data for experimental generation four in Chapter five. *n* = number of individuals in the experiment, DOD = day of death (if individuals died during the 30 day experimental period), MNO = mean number of offspring, B = brood, the range is given in days.

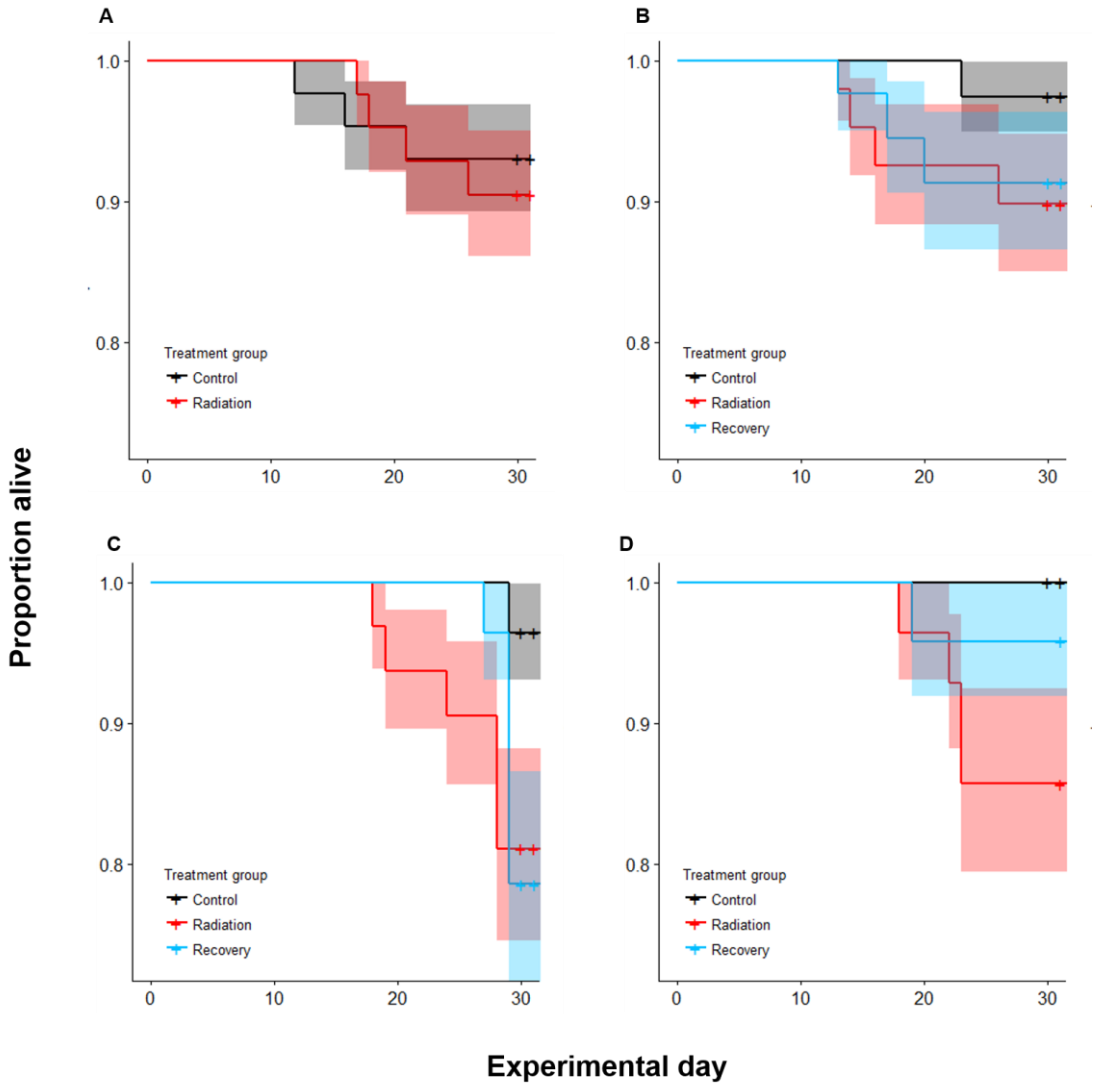
Treatment	*n*	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3	MNO B4	Range B4
Control	42	23	10 - 29	5	1 - 10	8	2 - 17	15	1 - 20	21	10 - 35
Radiation	36	24	18 - 28	5	1 - 11	10	4 - 16	16	8 - 36	17	10 - 24
Recovery	40	29	27 - 29	5	2 - 14	9	1 - 16	16	7 - 30	15	3 - 24

Appendix H4: Summary data for experimental generation six in Chapter five. *n* = number of individuals in the experiment, DOD = day of death (if individuals died during the 30 day experimental period), MNO = mean number of offspring, B = brood, the range is given in days.

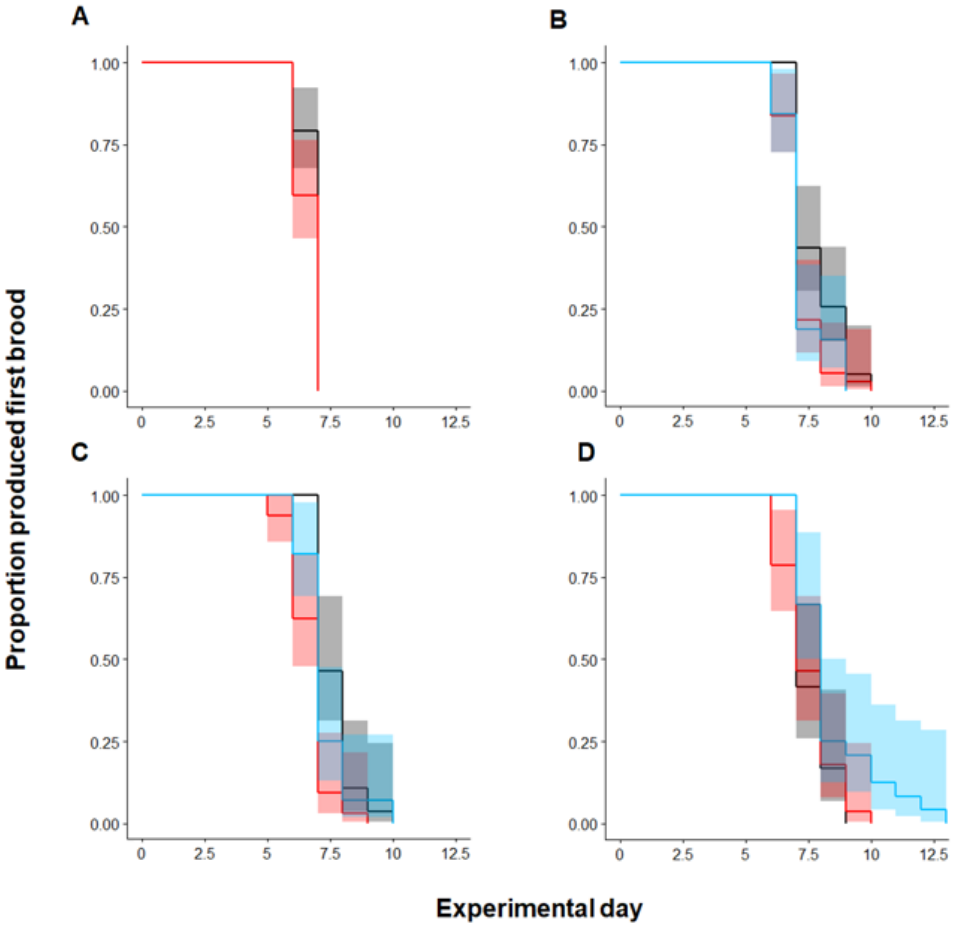
Treatment	*n*	Mean DOD	Range DOD	MNO B1	Range B1	MNO B2	Range B2	MNO B3	Range B3	MNO B4	Range B4
Control	42	34	34 - 34	4	1 - 12	8	2 - 15	12	6 - 20	15	6 - 26
Radiation	32	22	18 - 23	5	1 - 11	9	5 - 16	11	5 - 28	12	2 - 22
Recovery	39	19	19 - 19	5	2 - 9	8	2 - 16	11	5 - 15	13	4 - 30

Appendix I

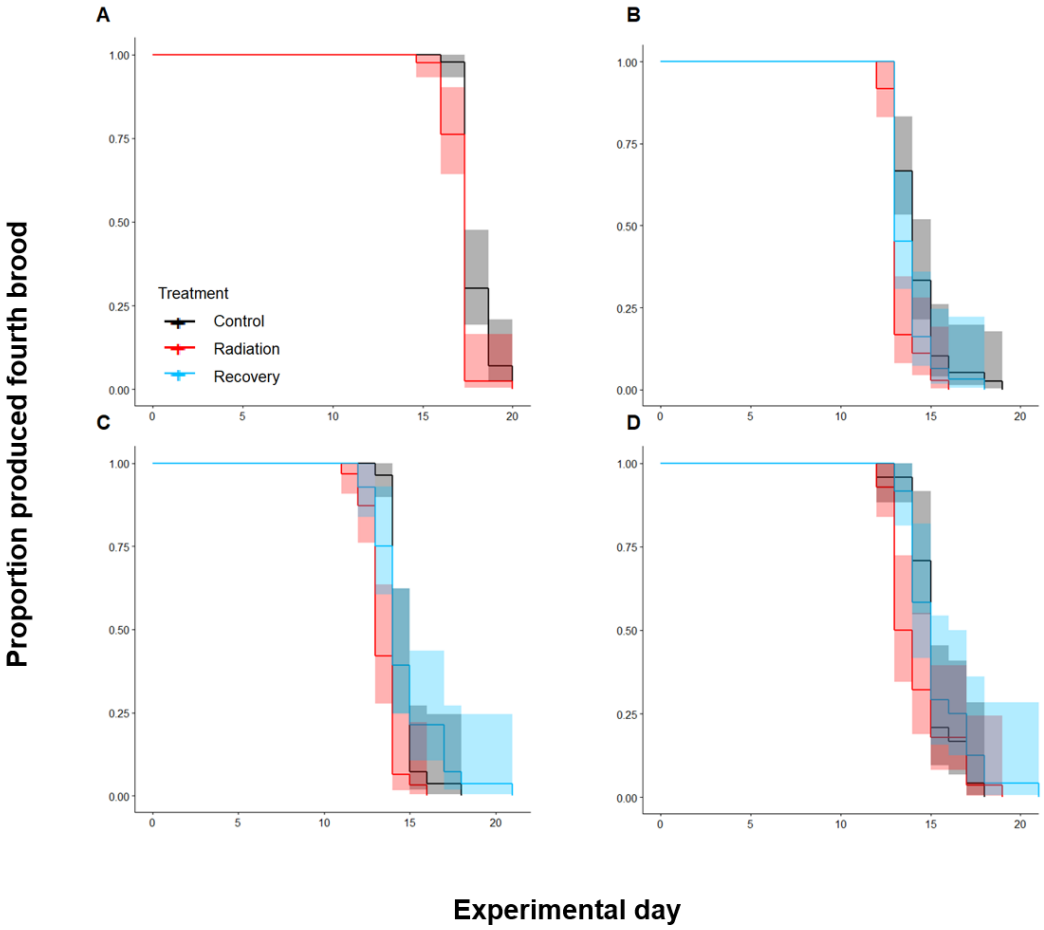
Survival in the control treatment varied significantly across generations (COXPH: χ^2_3= 12.19, $p < 0.01$). Similarly, there was significant variation in the total number of offspring produced (GLM: χ^2_3= 229.11, $p < 0.0001$), the day of production of the first and fourth broods (first brood, COXPH: χ^2_3= 34.71, $p < 0.0001$. Fourth brood, COXPH: χ^2_3= 37.72, $p < 0.0001$) and in age-specific fecundity (LME: χ^2_3= 25.8, $p < 0.001$) across generations in the control treatment. I therefore analysed each generation separately throughout Chapter five.



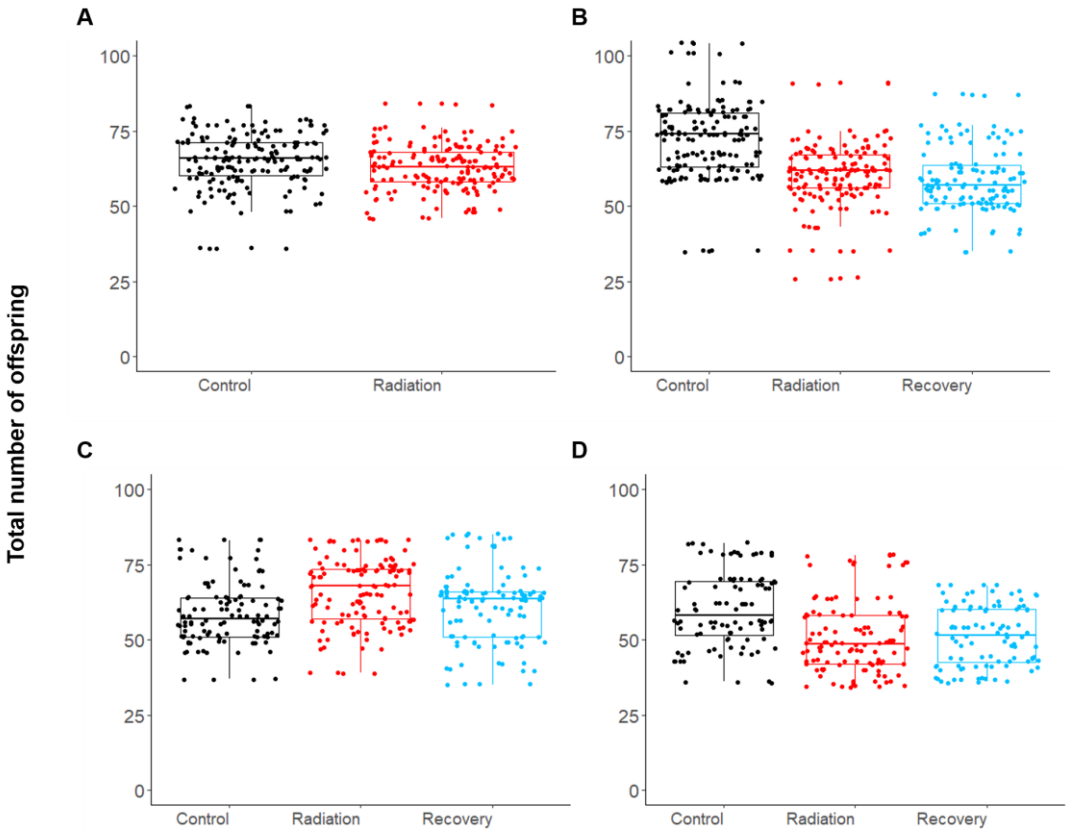
Appendix J: Survival estimates with 95% confidence intervals for each treatment group in generations (A) one, (B) two, (C) four and (D) six.



Appendix K: Effect of treatment group on time until first brood release in generations (A) one, (B) two, (C) four and (D) six. Shaded regions denote ± 95% confidence intervals [CIs]).



Appendix L: Effect of treatment group on time until the fourth brood release in generations (A) one, (B) two, (C) four and (D) six. Shaded regions denote ± 95% confidence intervals [CIs]).



Appendix M: Boxplot showing the total number of offspring produced by each treatment group over five clutches in generations (A) one, (B) two, (C) four and (D) six. The box shows the upper and lower quartiles within the data and the line within each box shows the median value. The lines outside of each box show the range of the data. The dots show the raw data values.

Appendix N: Total number of individuals that died in each treatment in each generation.

Generation	Control	Radiation	Recovery
1	4	4	-
2	1	4	4
4	2	6	6
6	0	4	1
Total	7	18	11

Appendix O: Total number of lineages lost from each treatment in each generation.

Generation	Control	Radiation
1	-	-
2	5	6
4	10	5
6	7	1
Total	22	12