Ecotoxicology (2013) 22:815–824
DOI 10.1007/s10646-013-1088-3

Parental exposure to methyl methane sulfonate of three-spined stickleback: contribution of DNA damage in male and female germ cells to further development impairment in progeny

R. Santos · M. Palos-Ladeiro · A. Besnard · J. Reggio · E. Vulliet · J. M. Porcher · S. Bony · W. Sanchez · A. Devaux

Accepted: 21 May 2013 / Published online: 7 June 2013

Abstract Data regarding the link between DNA integrity of germ cells and the quality of progeny in fish exposed to genotoxicant are scarce although such information is of value to understand genotoxic effects of contaminants in aquatic fauna. This work aimed at studying the consequences of a parental exposure during the breeding season on offspring quality in three-spined stickleback. After in vivo exposure of adult fish to methyl methane sulfonate, a model alkylating compound, a clear increase in DNA damage was observed in erythrocytes of both genders, here used as a biomarker of exposure. MMS exposure significantly affected sperm DNA integrity but neither female fecundity nor fertilization success. In order to understand the contribution of each sex to potential deleterious effects in progeny due to parental exposure, mating of males and females exposed or not to MMS, was carried out. Exposure of both males and females or of males alone led to a significant increase in both mortality during embryo–larval stages and abnormality rate at hatching that appeared to be sensitive stages. Thus, in accordance with recent studies carried out in other freshwater fish species, such development defects in progeny were clearly driven by male genome, known to be devoid of DNA repair capacity in spermatozoa. The next step will be to investigate the link between DNA damage in stickleback sperm and reproductive impairment in natural populations exposed to complex mixture of genotoxicants.

Keywords Stickleback · Genotoxicity · Comet assay · Germ cell · Reproduction · Offspring quality

R. Santos · S. Bony · A. Devaux (✉)
Université de Lyon, UMR 5023 LEHNA, 69100 Villeurbanne, France
e-mail: alain.devaux@entpe.fr

R. Santos · M. Palos-Ladeiro · J. M. Porcher · W. Sanchez
INERIS, Unité d'écotoxicologie in vitro et in vivo, BP 2, 60550 Verneuil en Halatte, France

A. Besnard
Centre d'Ecologie Fonctionnelle et Evolutive (UMR 5175), EPHE, Biogéographie et Ecologie des Vertébrés, campus CNRS, 1919 route de Mende, 34293 Montpellier cedex 5, France

J. Reggio · E. Vulliet
Département Service Central d'Analyse, Institut des Sciences Analytiques, UMR 5280, 5 rue de la Doua, 69100 Villeurbanne, France

S. Bony · A. Devaux
INRA, USC IGH, ENTPE, 69518 Vaulx en Velin, France

Introduction

Many surface waters have been reported to be contaminated with genotoxic substances through agricultural practices, as well as through industrial and urban activities dispersing a wealth of both organic and inorganic compounds responsible for serious toxic threats (Depledge and Galloway 2005). Identification of genetic risks related to environmental genotoxicants is a crucial point as mutations are known to be involved in the onset of a large array of defects such as developmental impairment or cancer occurrence (Bickham and Smolen 1994; Shugart and Theodorakis 1994). The study of genotoxic effects in germ cells of aquatic organisms coupled with consequences on population dynamics has historically been considered as a priority (Würgler and Kramers 1992; Anderson and Wild 1994; Depledge 1998). Since the publication of the International Program on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing (Ashby et al. 1996),

guidelines for mutagen testing in humans were developed and recently updated by the experts of the World Health Organization (WHO). A weight of evidence approach based on a combination of in vitro and in vivo assays in both somatic and germ cells assessed major endpoints of genetic damage such as gene mutation, clastogenicity or aneuploidy (Dearfield et al. 2002; Eastmond et al. 2009). If genetic damage occurs in somatic cells, deleterious effects are restricted to the exposed organisms but when it affects germ cells, it may increase the risk of progeny defects as clearly pointed out in humans and aquatic species (Shugart and Theodorakis 1998; Dearfield et al. 2002). The pioneer work published by Evenson et al. (1980) in mammals showed a significant relationship between human or bull sperm DNA fragmentation and loss of fertility. Then, a plethora of studies carried out in humans have indicated that sperm DNA damage was closely associated with male infertility, increased pregnancy loss, malformations or cases of childhood leukemia and autism (Aitken et al. 2009; Esteves et al. 2012). Regarding aquatic species, some studies have demonstrated a link between primary DNA damage in sperm and fertilization rate, hatching rate, and occurrence of morphological abnormalities in progeny of invertebrates (Lewis and Galloway 2009; Lacaze et al. 2011) and of fish exposed to various chemical stressors (Dietrich et al. 2005, 2010; Pérez-Cereales et al. 2010; Uren-Webster et al. 2010; Devaux et al. 2011).

Among the genotoxicity assays available nowadays, the comet assay (also called Single Cell Gel Electrophoresis) has been used historically to assess DNA damage in mammalian sperm (Singh et al. 1988) and its application to sperm of aquatic species in ecotoxicological studies has been more recently recommended to investigate the relationship between primary DNA damage in sperm and further detrimental effects in progeny (Jha 2008). Lewis and Ford (2012) suggested as one of the priority research areas in aquatic invertebrate ecotoxicology to study the sperm DNA damage link with fertilization success and subsequent development for a wide range of reproductive strategies and to up-scale it to assess population level effects. The alkaline version of the comet assay is considered as a sensitive, rapid, versatile and economic method based on a single-cell approach allowing to assess a large array of DNA damages including single and double strand breaks, DNA cross-links, alkali-labile sites and incomplete repair sites (Singh et al. 1988). The comet assay in sperm has already been included into strategy guidelines for the testing of chemicals for mutagenicity in humans (COM 2000; Baumgartner et al. 2012).

The three-spined stickleback (*Gasterosteus aculeatus*) is a biological model commonly used in ecotoxicology to assess adverse effects of pollutants for mechanistic studies in laboratory, and in the field using multi-biomarker approaches (Katsiadaki et al. 2002; Sanchez et al. 2005, 2008; Maunder et al. 2007; Pottinger et al. 2011; Katsiadaki et al. 2012). Recently, a relationship between the loss of DNA integrity in three-spined stickleback sperm and abnormal development in progeny has been shown after *ex vivo* exposure to methyl methane sulfonate (MMS), a model alkylating genotoxicant (Santos et al. 2013). As a complement to this last study, the aim of the present work was to address in the three-spined stickleback the consequences of in vivo parental MMS exposure during the breeding season on offspring quality. The protocol was designed to study the consequences of a parental genotoxic stress on progeny survival and development abnormalities, paying special attention to the contribution of the genetic load brought by each gender to the observed progeny defects.

Materials and methods

Fish origin, sex determination and exposure conditions

One-year old stickleback reared in an outdoor lotic mesocosm (INERIS, Verneuil en Halatte, France) were used. In October 2010, juvenile stickleback were transferred indoor into 500 l tanks with continuous water renewal. Fish were daily fed ad libitum with frozen bloodworms (Europrix, France) and reared at a water temperature of 13 ± 1 °C under natural photoperiods (from 10:14 to 11:13 h light: dark between November 2010 and March 2011). In March, 140 males and 200 females were sexed. Several morphological structures are different in male and female three-spined stickleback and among them, the head sexual dimorphism seems to be the most discriminating and robust one (Kitano et al. 2007; Aguirre and Akinpelu 2010). A mathematical model based on sexual dimorphism in the head morphology was developed to distinguish mature female and male. The difference between males and females was modeled with linear discriminant analysis using five metrics describing head morphology (De Kermoysan, unpublished data).

MMS [CAS number 66-27-3] and all other chemicals and reagents were purchased from Sigma-Aldrich chemicals (St Quentin Falavier, France). A total of 340 adult sticklebacks (44–60 mm length; 1.5–3.2 g body mass) were randomly dispersed into 20 l glass tank (10 females and 7 males per tank, total number of tanks n = 20) and a controlled photoperiod was applied from April to June 2011 (13:11–16: 8 h light:dark). As three-spined stickleback is a euryhaline fish species, the whole experiment was realized using 3 ‰ salted water (Regenit esco-salt tablets). After 10 days of acclimation, fish were exposed through water to MMS at three different concentrations (4 tanks per concentration, total

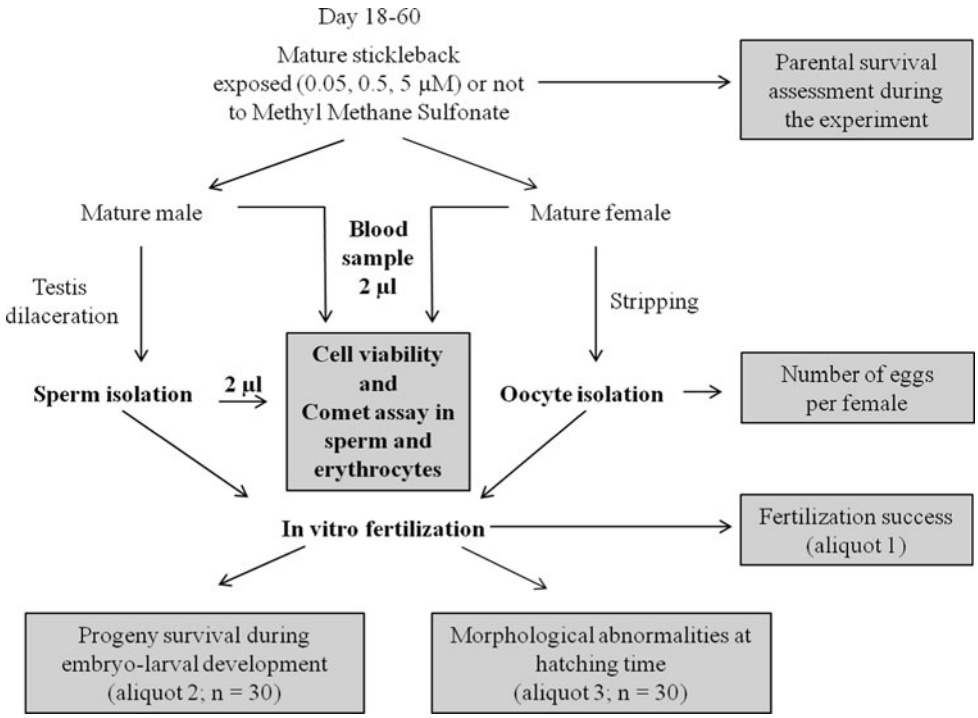

Fig. 1 Experimental design performed when fish were mature (females were gravid from 18 to 60 days of exposure). *Grey blocks* indicate endpoints measured on parents and progeny

number of exposed fish per concentration n = 68) or not exposed (8 tanks i.e. a total number of control fish n = 136). MMS was used as model genotoxicant since alkylating agents are thought to be the most potent and abundant genotoxic contaminants in the aquatic environment (Claxton et al. 1998). A preliminary experiment showed that time for 50 % mortality (LT_{50}) in stickleback exposed to 100 µM MMS was 29 days with reproductive, feeding and swimming behavior changes being observed after 2 weeks of exposure. Thus, far lower nominal MMS concentrations were used in the present experiment: 5, 0.5 and 0.05 µM and change in fish behavior was checked daily. A 50 mM MMS solution was prepared every day in distilled water and diluted in each tank to reach the nominal MMS concentration. Fish mortality was checked daily and water renewed (80 %) 1 h after feeding with frozen bloodworms (1 g/tank). Water was sampled in each tank twice during the experiment 2 h after water renewal. Water samples were frozen (−20 °C) and actual MMS concentrations were measured by capillary gas chromatography using flame ionization detection (Li 2004). During the course of experiment, water quality parameters were measured: pH 8.18 ± 0.07; temperature 15.94 ± 0.10 °C; 83.16 ± 3.10 % dissolved O_2 content; 3.70 ± 0.44 mS cm^{-1} conductivity; 15.0 ± 1.5 mg l^{-1} NO_3^-, 0.06 ± 0.06 mg l^{-1} NO_2^-, total ammonia being not detected (detection limit 0.25 mg l^{-1}).

For all MMS exposure concentrations, the first females were gravid and ready for stripping after 18 days of exposure. From day 18 to 60, mature females and males were used to realize fertilization. Then fertilization success, DNA damage in sperm and erythrocytes, progeny survival and abnormalities were assessed (Fig. 1). Males and females were daily checked for sexual maturity and mating was carried out as follows: mating of unexposed males and females, mating of males and females exposed to the same MMS concentration, and for each MMS concentration mating of exposed females and unexposed males and mating of unexposed females and exposed males. As a whole, 10 different mating conditions were applied.

Cell collection and fertilization

Blood was sampled in both males and females in order to measure DNA damage in erythrocytes, here only used as a biomarker of exposure to MMS. Primary DNA damage in germ cells was only investigated in males since assessment of DNA damage in stickleback ovocyte is to date not feasible in this species. Fish were anesthetized using tricaine mesilate (MS 222: 70 mg l^{-1}). Pygostyle was severed and 2 µl of blood were sampled in the caudal vein and 100× diluted in 1 % heparinized Hank's Balanced Salt Solution (HBSS). Testes were then immediately dissected out and were gently dilacerated in 350 µl of cold HBSS in order to collect mature spermatozoa. Two µl of sperm suspension were mixed with 198 µl of HBSS and were stored on ice as well as erythrocyte suspensions, until processing the comet assay (see below). Eggs were obtained by gentle abdominal stripping of females and spawnings were kept separate. According to the mating scheme, the remaining sperm suspension and freshly stripped eggs were mixed to perform fertilization as follows.

Each clutch was gently mixed with 198 µl sperm suspension and let to fertilize for 2 min at 20 °C. Eggs were then washed with HBSS, then with a 0.15 % hypochlorite solution and finally with a 0.7 % NaCl solution (5 min each washing). After 2 h at 20 °C, the first cell cleavages were observed and eggs were then counted to assess fecundity. Each clutch was divided in three aliquots to study fertilization success, embryo–larval survival and morphological abnormalities in progeny at hatching stage (Fig. 1). Eight to nine fertilizations were performed for each of the 10 mating conditions, resulting in a total number of n = 85 fertilizations.

Comet assay in sperm and erythrocytes

Sperm and erythrocytes were diluted in HBSS to adjust cell density. Cell viability was checked using the Trypan blue exclusion method for erythrocytes and with the LIVE/DEAD® sperm viability kit (Molecular Probes, L-7011) using SYBR14 dye and propidium iodide for spermatozoa. All the cell suspensions exhibited over 90 % of viability. Cell suspensions were equally mixed with 1 % low melting agarose prepared in HBSS (37 °C) and 100 µl of the final cell suspension were spread on a coated slide and covered with a coverslip. Slides were cooled for 10 min at 4 °C for agarose polymerization. Thereafter, the coverslip was removed and the slides were immersed into a freshly prepared lysing solution for 1 h at 4 °C in the dark (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1 % Triton X-100 and 10 % DMSO, pH 10). After lysis, slides were carefully placed in a horizontal electrophoresis tank filled with a freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). DNA was allowed to unwind for 40 min at 4 °C and then, electrophoresis was performed under 0.66 V cm^{-1} for 24 min. After electrophoresis, the slides were washed 3 times for 5 min with a neutralization buffer (0.4 M Tris–HCl, pH 7.5). From the lysis to the neutralization step, the assay was performed under dim yellow light or in the dark to prevent artifactual DNA damage. Finally, the slides were dried for 15 min in absolute ethanol. After staining with 0.02 % ethidium bromide solution, DNA damage of 100 randomly selected cells per slide was analyzed using an Axioskop epifluorescence microscope (Zeiss, Germany) and an image analysis system (Comet IV software, Perceptive Instruments Ltd., UK). Among the comet parameters, tail intensity (percentage in tail DNA) described as the most relevant one, was chosen (Collins 2004).

Fertilization success, morphological abnormalities at hatching and progeny survival

As previously indicated, each clutch was divided in 3 aliquots. An egg aliquot was maintained in freshwater in a Petri dish filled with freshwater for 24 h at 20 ± 0.5 °C and observed using a stereomicroscope (Leica MZ 12.5) in order to evaluate fertilization success (aliquot 1). Eggs that did not reach the epiboly stage (Swarup 1958) were considered as unfertilized since parthenogenetic embryos usually die within a few hours (Kopeika et al. 2004) and zygotic transcription initiation occurs during the "maternal embryo transition" (MET) which occurs in fish at the mid-blastula stage (Kimmel et al. 1995; Bobe and Labbé 2010). The very early steps of embryonic development until MET rely on gene products (maternal mRNAs and proteins) supplied to the embryo by the mother and could be impacted on by non-genetic factors such as environmental variables (e.g. photoperiod) (Bonnet et al. 2007). 2 h post-fertilization, two other aliquots of 30 fertilized eggs were gently placed in 2 flow-through water incubators to further study embryo–larval survival (aliquot 2) and morphological abnormalities in embryos at hatching (aliquot 3). Water temperature was maintained at 20 ± 0.5 °C during embryogenesis. Dead embryos were counted and removed every day until hatching that occurred 7 days after fertilization. At hatching, surviving larvae from aliquot 3 were collected to evaluate morphological abnormalities previously described in this species using a stereomicroscope Leica MZ 12.5 (Santos et al. 2013). Surviving larvae of aliquot 2 were gently collected, counted and immediately placed in a 2 l net with a continuous water renewal. Larvae were fed twice a day with freshly cultured artemia nauplii, weekly counted and reared until 28 days post-fertilization (DPF). To resume, progeny survival was investigated during embryogenesis at 5, 7 (hatching), 14, 21 DPF and the progeny was sacrificed at 28 DPF corresponding to the end of stickleback larval development at 20 °C (Swarup 1958).

Statistical analysis

Since tail intensity measured in spermatozoa and erythrocytes followed a Gaussian distribution after Arcsin square root transformation, we investigated the relationship between MMS concentration in interaction with exposure duration on (1) erythrocyte DNA damage, (2) spermatozoa DNA damage by using Generalized Linear models with Gaussian error on arcsin square root transformed data. We also tested whether the MMS concentration and exposure duration had an impact on (3) fecundity using Generalized Linear models with negative binomial distribution. Last, we explored the impact of MMS concentration, exposure duration and their interaction on (4) the survival of exposed adult sticklebacks, (5) the fertilization success, (6) morphological abnormalities in progeny and (7) progeny survival by using Generalized Linear Mixed Models (GLMM) with a binomial error. A 'female' or a 'mating' random effect were added to the model in order to deal with the

pseudo-replication induced by the fact that several oocytes stemmed from the same female (female effect) and several larvae from the same genitors (mating effect). To test the effects of MMS concentration and exposure duration we systematically fitted five models (null, with MMS concentration only, with exposure duration only and with MMS concentration and exposure duration in addition or in interaction). Among these five models, the model chosen in the present study fitted the best the data according to Akaike Information Criterion (AIC) as described in Santos et al. (2013).

Analysis of progeny survival was made with Kaplan–Meier survival curves allowing identifying whenever larvae died during embryo larval development. Data statistics are reported as mean ± SE. All analyses were performed with R 2.13.1 (R Development Core Team 2011).

Results

Effect of MMS exposure on fertilization success and on erythrocyte and spermatozoa DNA integrity

Stickleback were exposed to actual MMS concentrations of 8.32 ± 1.23 µM (5 µM nominal concentration), 0.78 ± 0.00 µM (0.5 µM nominal concentration) and 0.065 ± 0.005 µM (0.05 µM nominal concentration) respectively. MMS concentrations measured in water were higher than expected probably due to semi-static conditions requiring partial water renewal. A total of 5.29 % of adult fish died due to experimental conditions. No significant relationship was shown between adult mortality and MMS exposure concentration as the fitted model had higher AIC than the null model (AIC = 145.01 and 142.82 respectively, $F_{3,336} = 1.27$, $p = 0.28$). No significant relationship between fertilization success and (i) oocyte origin (i.e. the female), and (ii) male MMS exposure concentration or (iii) DNA damage in sperm was observed (null model: AIC = 1465.2; female exposure: AIC = 1469.1, $X^2_3 = 2.12$, $p = 0.55$; male exposure: AIC = 1468.7, $X^2_3 = 2.48$, $p = 0.48$; sperm DNA damage: AIC = 1467.2, $X^2_1 = 0.05$, $p = 0.82$). MMS exposure of stickleback did not affect female fecundity expressed as the number of eggs produced per female, here n = 111 ± 26 eggs per female in average (fitted model: AIC = 821.3; null model: AIC = 821.6, $X^2_3 = 6.25$, $p = 0.10$).

After 18 days of exposure, DNA damage significantly increased in erythrocytes of fish exposed to 0.5 and 5 µM MMS compared to the control ($p = 0.02$ and $p < 0.01$ respectively). Within the 42 days of the experiment, the level of DNA damage in erythrocytes decreased significantly in control and marginally in 0.05 µM groups from 15.96 ± 1.70 to 8.90 ± 0.87 % and 17.98 ± 1.71 to 13.34 ± 1.30 % (slope estimate $\beta = -0.003 \pm 0.001$, $t = -4.03$, $p < 0.01$ and slope estimate $\beta = -0.002 \pm 0.001$, $t = -1.88$, $p = 0.06$, respectively; Fig. 2a). At 0.5 µM MMS, the level of DNA damage increased significantly reaching 49.94 ± 3.87 % tail intensity after 60 days (slope estimate $\beta = -0.007 \pm 0.001$, $t = 6.29$, $p < 0.01$; Fig. 2a). Concerning the highest MMS concentration, between 18 and 60 days of exposure, DNA damage value in erythrocytes led to a saturated signal (>95 % of tail intensity) although cell viability remained higher than 90 %. A high correlation between DNA damage measured in male and female erythrocytes was underlined ($R^2 = 0.96$, $p < 0.01$) and no significant difference in DNA damage level was shown between both sexes whatever the MMS concentration.

No change in male behavior was noticed, whatever the treatment. Basal DNA damage in control sperm remained stable (17.21 ± 0.79 %) all along the experiment (slope estimate $\beta = -0.0007 \pm 0.0009$, $t = -0.757$, $p = 0.45$; Fig. 2b). A significant increase in DNA damage in sperm of fish exposed to 0.05 µM (slope estimate $\beta = 0.003 \pm 0.001$, $t = 3.018$, $p < 0.01$) and 0.5 µM MMS (slope estimate $\beta = 0.016 \pm 0.001$, $t = 12.09$, $p < 0.001$) was shown between day 18 and 60, tail intensity values increasing from 13.88 ± 3.25 to 28.51 ± 2.09 % and 33.51 ± 3.14 to 87.04 ± 3.14 % respectively (Fig. 2b). After exposure to 5 µM MMS, DNA damage in stickleback sperm was significantly higher than in control all along the exposure (slope estimate $\beta = 1.059 \pm 0.07$, $t = 14.61$, $p < 0.001$) and the signal remained saturated (>95 % of tail intensity) during the experiment. DNA damage in erythrocytes was highly correlated with DNA damage measured in sperm ($R^2 = 0.89$, $p < 0.01$).

Progeny survival during embryo–larval development

After male exposure, progeny death probability in control remained stable and lower than 0.10 all along the experiment (Fig. 3a). Male exposure to MMS induced a decrease in progeny survival according to Kaplan–Meier survival analyses. Cumulative survival probability at the end of embryo–larval development (28 DPF) was 0.91 ± 0.01 in control, 0.73 ± 0.03 at 0.05 µM, 0.82 ± 0.02 at 0.5 µM and 0.47 ± 0.03 at 5 µM, the highest MMS concentration. Among the 960 larvae stemming from control or exposed male sticklebacks, 245 (37.9 %) died during embryo–larval stages mainly between 5 and 7 DPF (hatching). A total of 22 % of larvae died before 5 DPF, 25.3 % during the first week after hatching (>7 DPF) and 14.6 % died from 14 to 28 DPF. Taking into account MMS concentration in interaction with exposure duration, the fitted model (AIC = 876.10 and null model AIC = 923.07; Fig. 3b) underlined a significant and dramatic decrease in survival probability for larvae issued

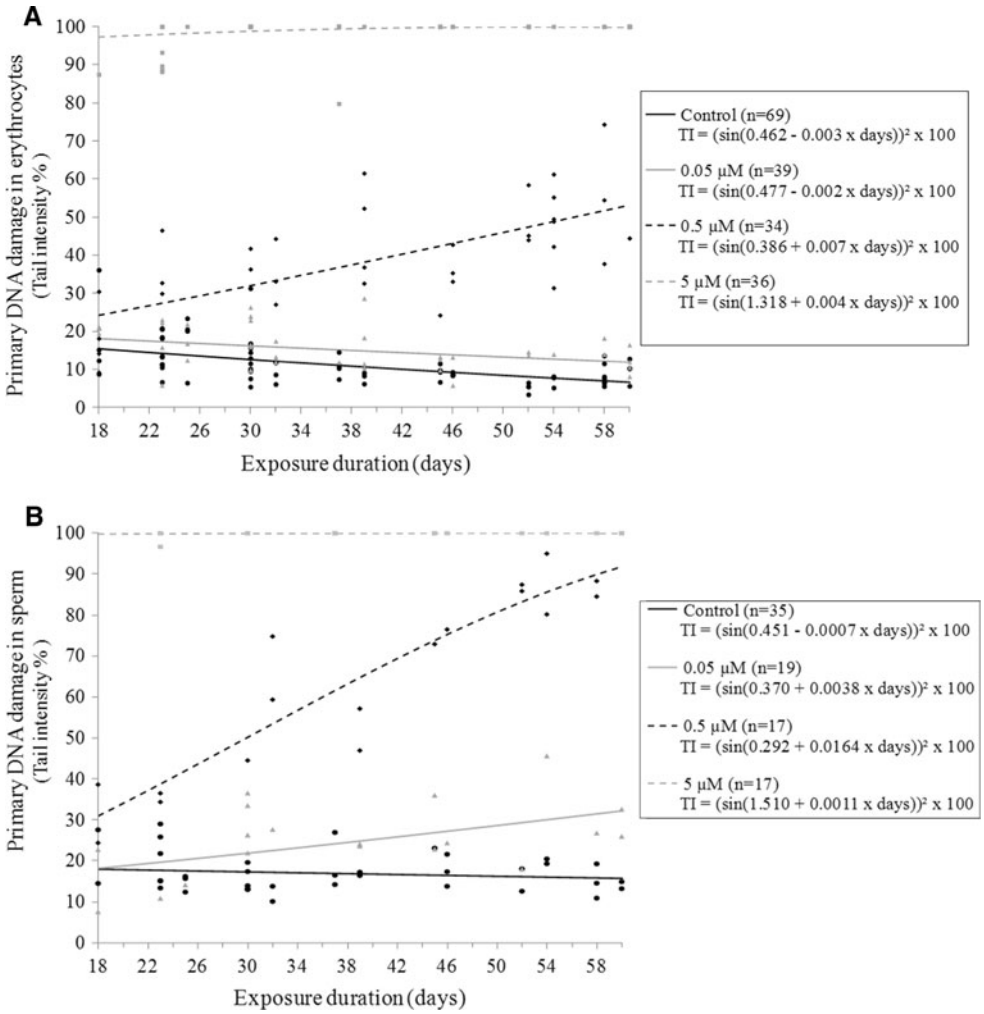

Fig. 2 Primary DNA damage expressed as tail intensity percentage (TI) in erythrocytes of both male and female stickleback (**a**) and in sperm of male stickleback (**b**) exposed from 18 to 60 days to methyl methane sulfonate (control: *black line*; 0.05 µM: *grey line*; 0.5 µM: *black dashed line*; 5 µM: *grey dashed line*; fitted model AIC = −330.93 and null model AIC = 235.45 for erythrocytes, fitted model AIC = −195.09 and null model AIC = 114.26 for sperm). For each condition of exposure, the plot is fitted to raw data from individual animals where n = the number of fish used for the curve fit

from long paternal duration exposure at the highest MMS concentration (slope estimate $\beta = -0.10 \pm 0.01$, $t = -6.99$, $p < 0.01$; 0.14: probability of survival during embryo–larval development after 8 weeks of paternal exposure). At lower MMS concentrations (0.05 and 0.5 µM), progeny survival probability was significantly lower after 18 days of exposure compared to the control (respectively slope estimate $\beta = -2.11 \pm 0.51$, $t = -4.15$, $p < 0.01$ and slope estimate $\beta = -1.41 \pm 0.51$, $t = -2.79$, $p < 0.01$) and tended to increase according to paternal exposure duration (respectively slope estimate $\beta = 0.030 \pm 0.012$, $t = 2.34$, $p = 0.02$, slope estimate $\beta = 0.026 \pm 0.015$, $t = 1.81$, $p = 0.07$ respectively).

After female exposure, progeny death probability in control remained stable and lower than 0.10 (0.091 ± 0.01) all along the experiment (Fig. 3c). Cumulative survival probability at the end of embryo–larval development (28 DPF) was respectively 0.91 ± 0.01 in control, 0.83 ± 0.02 at 0.05 µM, 0.82 ± 0.02 at 0.5 µM and 0.83 ± 0.02 at 5 µM MMS. Among the 960 larvae stemming from control or exposed female stickleback, 132 (13.75 %) died during embryo–larval stages (5–7 DPF). A total of 17.4 % died before 5 DPF, 21.2 % between 5 DPF and hatching (7 DPF), 31.8 % before 14 DPF and 27.9 % during the last two weeks of larval development (14 DPF to 28 DPF). Whatever the MMS concentration no significant difference in mortality probability was noticed (AIC = 789.74 and null model AIC = 790.95; Fig. 3d).

After exposure of both parents, cumulative survival probability at the end of embryo–larval development was 0.91 ± 0.01 in control, 0.88 ± 0.01 at 0.05 µM, 0.82 ± 0.02 at 0.5 µM and 0.43 ± 0.03 at 5 µM MMS (Fig. 3e). Among the 1080 larvae stemming from control or exposed male and female sticklebacks, 254 (23.51 %) of them died during embryo–larval stages (5–7 DPF). A total of 25.9 % died before 5 DPF and 35.8 % between 5 and 7 DPF (hatching stage). A total of 24.8 % died between hatching and 14 DPF and 9.0 % during the last two weeks of larval development. The fitted model (AIC = 904.68 and null model AIC = 949.07; Fig. 3f) underlined a significant and dramatic decrease in survival probability of larvae issued from long exposure duration

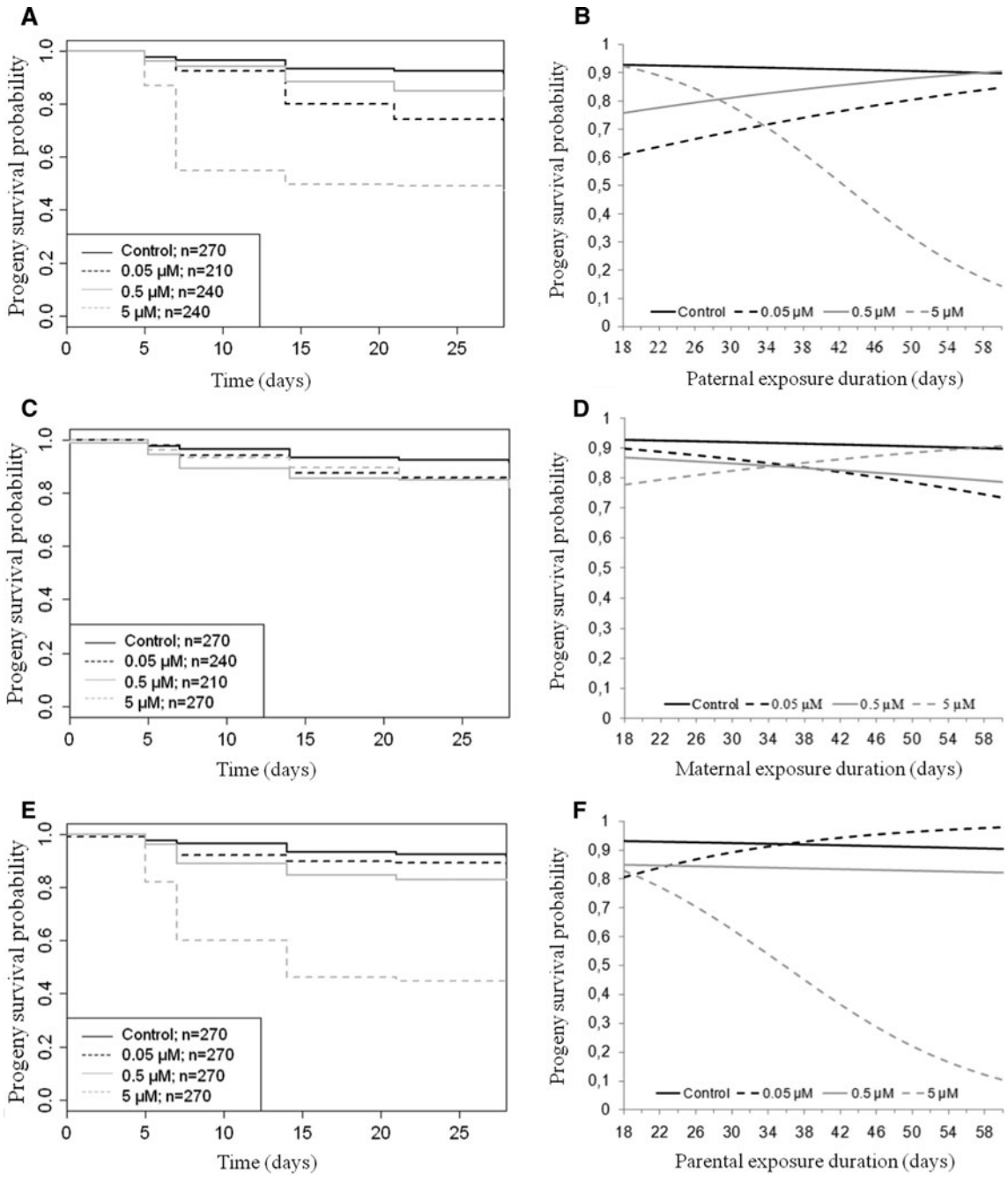

Fig. 3 Kaplan–Meier survival curves illustrating the probability of larvae mortality during the stickleback progeny embryo (from day 1 to hatching, day 7) and larval (from hatching, day 7–28) development (**a, c, e**) and survival mortality probability of progeny (**b, d, f**) stemming from paternal (**a, b**), maternal (**c, d**) or parental (**e, f**) methyl methane sulfonate exposure. The survival probability of the progeny was investigated analyzing the survival of 210–270 larvae per condition, each larva being considered as the statistical unit

of parents to the highest MMS concentration (5 µM, slope estimate $\beta = -0.089 \pm 0.017$, $t = -5.12$, $p < 0.001$), reaching a 0.10 value at the end of the exposure. At lower MMS concentrations (0.05 and 0.5 µM), probability was not significantly lower than in control (slope estimate $\beta = 0.058 \pm 0.029$, $t = 1.98$, $p = 0.06$ and slope estimate $\beta = -0.005 \pm 0.017$, $t = -0.30$, $p = 0.76$, respectively).

Morphological abnormalities of offspring at hatching stage

Morphological abnormalities were monitored in 2,670 larvae alive at hatching stage. A total of 157 larvae exhibited morphological abnormalities (5.9 %). Yolk sack edemas or cardiac edemas were frequent malformations (21.7 and 32 % respectively). Skeletal abnormalities (lordosis, kyphosis or

cranial malformation) were the most detected representing 41.5 % of malformed larvae. Finally, 4.8 % of other types of malformations affecting otic vesicle or yolk–sack were recorded. Significant relationships between MMS parental exposure according to sex and occurrence of morphological abnormalities in progeny were highlighted when males were exposed (7.07 % at 0.05 µM, $p = 0.02$; 2.76 % at 0.5 µM, $p = 0.26$ and 13.49 % at 5 µM, $p < 0.01$), when females were exposed (6.58 % at 0.05 µM, $p = 0.12$; 15.13 % at 0.05 µM, $p < 0.01$ and 6.56 % at 5 µM, $p = 0.04$) or when both genitors were exposed (5.14 % at 0.05 µM, $p = 0.27$; 7.37 % at 0.5 µM, $p < 0.01$ and 15.58 % at 5 µM, $p < 0.01$), while only 1.15 % of abnormalities were detected in control larvae. Fitted models were compared to null models by ANODEV and showed male, female and both parent effect (male exposure: AIC = 292.8, null model: AIC = 299.4; $X_1^2 = 12.67, p < 0.01$; female exposure: AIC = 378.7, null model: AIC = 381.9; $X_1^2 = 9.20, p = 0.02$; parental exposure: AIC = 381.1, null model: AIC = 402.03; $X_1^2 = 26.96, p < 0.01$).

Discussion

This study shows first that non-lethal exposure of adult stickleback to MMS used as a model alkylating genotoxicant does not influence the reproductive behavior of male stickleback described by Wooton (1976). Second, MMS treatment has no deleterious effect on fecundity (considered here as the number of eggs spawned) and on fertilization success. However, results show that parental exposure to MMS gives rise to direct DNA damage in gametes further leading to a decrease in progeny survival and to the occurrence of morphological abnormalities in fish larvae. In the present study, MMS exposure increased significantly DNA damage in both male and female erythrocytes and in sperm, demonstrating a clear genotoxic impact on both genders. Erythrocyte and sperm DNA was highly damaged after fish exposure to the highest MMS concentration reaching a tail intensity value >95 % (Fig. 2a, b) although no decrease in viability of both cell types and sperm fertilizing ability, as well as no increase in fish mortality were observed along the 70-day experiment. Fish erythrocyte DNA as well as highly condensed DNA in spermatozoa are considered as inert chromatin, inactive regarding DNA synthesis (Lemke et al. 1999). The general trend towards a higher DNA damage level in spermatozoa compared to erythrocytes which has been observed all along the experiment could be explained by the erythrocyte turn over ranging from 1 to 3 months in fish (Udroiu 2006). In the present study DNA damage observed in sperm after MMS treatment did not decrease fertilizing ability and, if not or incompletely repaired by the zygote, may impact offspring development and survival. A similar effect has been already demonstrated in rats exposed to cyclophosphamide (Trasler et al. 1985), and more recently in fish (Devaux et al. 2011; Santos et al. 2013) and aquatic invertebrates after MMS exposure (Lewis and Galloway 2009). Other studies have shown that DNA-damaged sperm in fish could affect fertilizing capacity, likely to induce further embryo developmental defects as highlighted in trout after exposure to UV radiation, hydrogen peroxide or after sperm cryopreservation (Dietrich et al. 2005; Pérez-Cereales et al. 2010). But one must keep in mind that some chemicals can compromise fertilization success through mechanisms other than an increase in DNA damage in germ cells, depending on the mode of action of the compound (Uren-Webster et al. 2010). These authors demonstrated that exposure of male zebrafish to high concentrations of a phthalate widely used as a plasticizer did not affect sperm DNA integrity but caused a marked reduction in fertilization success through the disruption of early stages of spermatogenesis, without any abnormal development of progeny.

After MMS exposure of both parents, a significant impact on progeny survival was shown whatever the MMS concentration (Fig. 3). Embryo–larval stages are sensitive and represent crucial life stages as development defects especially during the organogenesis can be deleterious in the short term. Offspring mortality during embryo–larval development occurred here mainly the last two days before hatching. Those stages are particularly sensitive since morphogenesis of many vital organs is still in progress, as described in zebrafish and sea bass (Kimmel et al. 1995; Cucchi et al. 2012). Larvae mortality occurred also but to a lesser extent during the first week after hatching what corresponds to the larvae yolk–sack resorption and to the first exogenous food intake, also considered as critical larval stages in stickleback (Swarup 1958). Progeny survival patterns according to MMS concentration or exposure duration were similar when male stickleback or both genders were exposed. This result indicates that parental exposure to this genotoxicant induces development impairment in progeny mainly through paternal contribution. Whilst oocyte contains a variety of biotransformation and DNA repair enzymes that protect against environmentally induced damage, sperm is generally considered to have little or no capacity for DNA repair or anti-oxidant defense, at least regarding the last mature stages in charge of reproduction i.e. spermatozoa (Aitken et al. 2004). Thus spermatozoa are considered as a sensitive target toward genotoxic compounds, and sperm DNA integrity is pointed out as one of the major risk factor for abnormal development of progeny. Egami et al. (1983) have mated irradiated female medaka (*Oryzias latipes)* with non-irradiated males and have assessed hatchability of embryos. Authors showed a dose-response relationship between acute doses of irradiation

(which were lethal for fish less than 10 days later) and progeny survival. Nevertheless, lower chronic irradiation doses were not linked with a decrease in embryo survival probably due to oocyte DNA repair system activity, efficient enough to repair a lower level of DNA damage than those occurring after acute irradiation. Irradiation of males at similar chronic low doses resulted in deleterious effects in offspring leading to lethality. Our results are in accordance with this study. Indeed female stickleback exposure did not induce substantial decrease in progeny survival during the embryo–larval development even if the high DNA damage level measured in erythrocytes demonstrated an acute genotoxic stress in females.

A discrepancy was noted regarding the levels of sperm DNA damage and progeny mortality measured at the intermediate (0.5 μM) and at the low (0.05 μM) MMS concentrations (Fig. 3). This result could be related to the induction of DNA repair system in zygote as previously shown in mammals. As an example, in rat, poly (ADP-ribose) polymerase-1 (PARP-1) activation and H2AX histone protein (γH2AX) foci have been detected in pronucleus in response to paternal cyclophosphamide exposure (Barton et al. 2007; Grenier et al. 2010). γH2AX have been described in medaka embryos (Hidaka et al. 2010) and PARPs are among the most conservative enzymes of the DNA repair systems in vertebrates (Kopeika et al. 2004). In the present work, significant DNA damage in stickleback sperm at 0.5 μM MMS concentration could have induced such DNA repair processes in zygote. This is in line with the lower mortality in progeny observed after exposure to 0.5 μM MMS compared to the higher mortality at 0.05 μM MMS which in turn was not high enough to trigger zygote repair. Concerning the response toward the highest MMS concentration 5 μM, the large amount of primary DNA damage measured in sperm could have not efficiently been repaired due to the swamping of zygote DNA repair capacity, further inducing offspring defects.

In conclusion, results of this work provide a weight of evidence that stickleback offspring stemming from fish exposed to the alkylating compound MMS exhibit enhanced mortality and a clear increase in development abnormalities. Such a reproductive effect mainly driven by the paternal genome could represent a serious threat for the quality of progeny, in particular considering that fertilizing capacity is not affected by the genotoxicant as shown here. The present study has focused on endpoints such as embryo–larval survival and occurrence of development abnormalities at hatching in stickleback after a parental MMS exposure. This work could be complemented by studying offspring survival until sexual maturation and F1 reproduction success through long term laboratory MMS exposure. Additionally, the link between genotoxic damage in germ cells and reproductive impairment in natural populations of stickleback exposed to complex mixture of genotoxicants would deserve to be studied at the field scale.

Acknowledgments This work was supported by the French Ministry of Ecology and Sustainable Development (Programme 190 Ecotoxicology).The authors thank Cyril Turies, Vincent Lisiak and Benjamin Piccini for their help to build embryo incubators and larvae tanks. Thanks to Stephane Maestra and Patrick Nisole for helping to design the genotoxic waste water treatment system. Thanks to Goulwen de Kermoysan and Remy Baudoin for fish sexing. The authors thank anonymous reviewers for their constructive comments and valuable remarks that greatly improve the final version of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards Procedures described in the present paper were conducted in accordance with laws and regulations controlling animal experiments in France. All experimental protocols were approved by the ethical committee of the French Institute of Industrial Environment and Risks.

References

Aguirre WE, Akinpelu O (2010) Sexual dimorphism of head morphology in three-spined stickleback *Gasterosteus aculeatus*. J Fish Biol 77:802–821

Aitken RJ, Koopman P, Lewis SE (2004) Seeds of concern. Nature 432:48–52

Aitken RJ, De Iuliis GN, McLachlan RI (2009) Biological and clinical significance of DNA damage in the male germ line. Int J Androl 32:46–56

Anderson SL, Wild GC (1994) Linking genotoxic responses and reproductive success in ecotoxicology. Environ Health Perspect 102:9–12

Ashby J, Waters MD, Preston J et al (1996) IPCS harmonization of methods for the prediction and quantification of human carcinogenic/mutagenic hazard, and for indicating the probable mechanism of action of carcinogens. Mutat Res 352:153–157

Barton TS, Robaire B, Hales BF (2007) DNA damage recognition in the rat zygote following chronic paternal cyclophosphamide exposure. Toxicol Sci 100:495–503

Baumgartner A, Kurzawa-Zegota M, Laubenthal J et al (2012) Comet-assay parameters as rapid biomarkers of exposure to dietary/environmental compounds-An in vitro feasibility study on spermatozoa and lymphocytes. Mutat Res 743:25–35

Bickham JW, Smolen MJ (1994) Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ Health Perspect 102:25–28

Bobe J, Labbé C (2010) Egg and sperm quality in fish. Gen Comp Endocr 165:535–548

Bonnet E, Fostier A, Bobe J (2007) Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics 8:55

Claxton LD, Houk VS, Hughes TJ (1998) Genotoxicity of industrial wastes and effluents. Mutat Res 410:237–243

Collins AR (2004) The comet assay for DNA damage and repair: principles, applications and limitations. Mol Biotech 26:249–261

COM (2000) Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, Guidance on a Strategy for Testing of Chemicals for Mutagenicity, Ed.:

J. Parry, Crown copyright, UK Department of Health. http://www.advisorybodies.doh.gov.uk/com/guidance.pdf. Accessed 9 Mar 2013

Cucchi P, Sucré E, Santos R et al (2012) Embryonic development of the sea bass *Dicentrarchus labrax*. Helg Mar Res 66:199–209

Dearfield KL, Cimino MC, McCarroll NE et al (2002) Genotoxicity risk assessment: a proposed classification strategy. Mutat Res 521:121–135

Depledge MH (1998) The ecotoxicological significance of genotoxicity in marine invertebrates. Mutat Res 399:109–122

Depledge MH, Galloway TS (2005) Healthy animals, healthy ecosystems. Front Ecol Environ 3:251–258

Devaux A, Fiat L, Gillet C et al (2011) Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat Toxicol 101:405–411

Dietrich GJ, Szpyrka A, Wojtczak M et al (2005) Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology 64:1809–1822

Dietrich GJ, Dietrich M, Kowalski RK et al (2010) Exposure of rainbow trout milt to mercury and cadmium alters sperm motility parameters and reproductive success. Aquat Toxicol 97:277–284

Eastmond DA, Hartwig A, Anderson D et al (2009) Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS harmonized scheme. Mutagenesis 24:341–349

Egami N, Shimada A, Hamafurukawa A (1983) Dominant lethal mutation-rate after gamma-irradiation of the fish, *Oryzias latipes*. Mutat Res 107:265–277

Esteves SC, Hamada A, Kondray V et al (2012) What every gynecologist should know about male infertility: an update. Arch Gynecol Obstet 286:217–229

Evenson DP, Darzynkiewicz Z, Melamed MR (1980) Relationship of mammalian sperm chromatin heterogeneity to fertility. Science 210:1131–1133

Grenier L, Robaire B, Hales BF (2010) Paternal exposure to cyclophosphamide affects the progression of sperm chromatin decondensation and activates a DNA damage response in the prepronuclear rat zygote. Biol Reprod 83:195–204

Hidaka M, Oda S, Kuwahara Y et al (2010) Cell lines derived from a medaka radiation-sensitive mutant have defects in DNA double-strand break responses. J Radiat Res 51:165–171

Jha AN (2008) Ecotoxicological applications and significance of the comet assay. Mutagenesis 23:207–221

Katsiadaki I, Scott AP, Mayer I (2002) The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. Mar Envir Res 54:725–728

Katsiadaki I, Sanders M, Henrys P et al (2012) Field surveys reveal the presence of anti-androgens in an effluent-receiving river using stickleback-specific biomarkers. Aquat Toxicol 122–123:75–85

Kimmel CB, Ballard WW, Kimmel SR et al (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310

Kitano J, Mori S, Peichel CL (2007) Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). Copeia 2007:336–349

Kopeika J, Kopeika E, Zhang T et al (2004) Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (*Misgurnus fossilis*) embryos derived from cryopreserved sperm. Theriogenology 61:1661–1673

Lacaze E, Geffard O, Goyet D et al (2011) Linking genotoxic responses in *Gammarus fossarum* germ cells with reproduction impairment, using the Comet assay. Environ Res 111:626–634

Lemke MJ, Chiva M, Coyle B (1999) Variability of sperm nuclear basic proteins in the three-spined stickleback and related species of Gasterosteoidei. Comp Biochem Physiol 122:339–353

Lewis C, Ford AT (2012) Infertility in male aquatic invertebrates: a review. Aquat Toxicol 120–121:79–89

Lewis C, Galloway T (2009) Reproductive consequences of paternal genotoxin exposure in marine invertebrates. Environ Sci Technol 43:928–933

Li W (2004) Trace analysis of residual methyl methanesulfonate, ethyl methanesulfonate and isopropyl methanesulfonate in pharmaceuticals by capillary gas chromatography with flame ionization detection. J Chromatogr 1046:297–301

Maunder RJ, Matthiessen P, Sumpter JP et al (2007) Impaired reproduction in three-spined sticklebacks exposed to ethinyl estradiol as juveniles. Biol Reprod 77:999–1006

Pérez-Cereales S, Martinez-Pàramo S, Beirao M et al (2010) Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139:989–997

Pottinger TG, Cook A, Jürgens MD et al (2011) Effects of sewage effluent remediation on body size, somatic RNA: DNA ratio, and markers of chemical exposure in three-spined sticklebacks. Environ Int 37:158–169

R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org. Accessed 13 Jan 2012

Sanchez W, Palluel O, Meunier L et al (2005) Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environ Toxicol Pharmacol 19:177–183

Sanchez W, Katsiadaki I, Piccini B et al (2008) Biomarker responses in wild three-spined stickleback (*Gasterosteus aculeatus* L.) as a useful tool for freshwater biomonitoring: a multiparametric approach. Environ Int 34:490–498

Santos R, Palos-Ladeiro M, Besnard A et al (2013) Relationship between DNA damage in sperm after *ex vivo* exposure and abnormal embryo development in the progeny of the three-spined stickleback. Reprod Toxicol 36:6–11

Shugart LR, Theodorakis C (1994) Environmental genotoxicity: probing the underlying mechanisms. Environ Health Perspect 102:13–17

Shugart LR, Theodorakis C (1998) New trends in biological monitoring: application of biomarkers to genetic ecotoxicology. Biotherapy 11:119–127

Singh NP, McCoy MT, Tice RR et al (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Swarup H (1958) Stages in the development of the stickleback *Gasterosteus aculeatus* (L.). J Embryol Exp Morphol 6:373–383

Trasler JM, Hales BF, Robaire B (1985) Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. Nature 316:144–146

Udroiu I (2006) The micronucleus test in piscine erythrocytes. Aquat Toxicol 79:201–204

Uren-Webster TM, Lewis C, Filby AL et al (2010) Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquat Toxicol 99:360–369

Wooton RJ (1976) The biology of the sticklebacks. Academic Press, London

Würgler FE, Kramers PGN (1992) Environmental effects of genotoxins (ecogenotoxicology). Mutagenesis 7:321–327