The influence of environmental UVB radiation on growth, immunity, behavior and sexually selected traits in the three-spined stickleback (*Gasterosteus aculeatus*)

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Content

General introduction

Facing changing environments

Changing environmental conditions can be caused by multiple factors, and especially the anthropogenic-induced impact on environmental conditions is well documented and has serious effects on animal communities (Walther *et al.*, 2002; Bradshaw and Holzapfel, 2008; Turner *et al.*, 2020). Thus, Palumbi (2001) describes humans as the greatest evolutionary force and anthropogenic caused or changed selection pressures are prevalent and often go beyond those by natural changes (Reznick and Ghalambor, 2001; Boivin *et al.*, 2016).

Changing environments often result in stressful conditions followed by responses of organisms aiming to attenuate negative consequences. Thus, animals may respond by altered behavior, morphology, physiology or even genetics, with consequences for population dynamics and community structure (reviewed in Sergio *et al.*, 2018). Genetic responses, i.e. evolutionary adaptations, occur e.g. by selection of emerging mutations (reviewed in Hoffmann and Sgro, 2011), whereas behavioral responses are often expressed by migration, i.e. escaping the stressful conditions (reviewed in Parmesan, 2006; Radchuk *et al.*, 2019). However, migration is often restricted e.g. by specific habitat requirements, natural or anthropogenic barriers (Butikofer *et al.*, 2019).

When migration options are limited, coping with the stressful conditions often results in phenotypic plasticity, i.e. the expression of alternative phenotypes based on one genotype, which represents a non-genetic response and is often individual's first reaction to environmental changes (Bonamour *et al.*, 2019; Kelly, 2019; Scheiner *et al.*, 2020). Phenotypic plasticity can include morphological, physiological and behavioral changes at the individual level (Ghalambor *et al.*, 2007; Gibert *et al.*, 2019; Scheiner *et al.*, 2020) but may also include transgenerational effects, i.e. transgenerational plasticity (TGP), mostly based on maternal effects (e.g. Shama *et al.*, 2014; Shama *et al.*, 2016). Thus, TGP can mitigate adverse effects of environmental changes and enhance offspring fitness (reviewed in Donelson *et al.*, 2018). In contrast, stress in parents may also cause changes in offspring that reduce fitness, i.e. maladaptive transgenerational plasticity, which may ultimately result in extinction (Sultan *et al.*, 2009; Guillaume *et al.*, 2016). Though, both TGP and within-generation plasticity are supposed to facilitate rapid responses to environmental changes (e.g. Fuxjäger *et al.*, 2019). However, the expression and preservation of phenotypic plastic traits is often linked to costs and simply holding the ability of expressing plastic responses could affect fitness (DeWitt *et*

al., 1998; Relyea, 2002). Furthermore, phenotypic plasticity can also be limited by e.g. imperfect sensory mechanisms or conditions impairing the assessment of environmental conditions (reviewed in Auld *et al.*, 2010).

In general, while phenotypic plasticity is a major mechanism of responses to changing environments, it can only be adaptive to a certain degree when environmental change is rapid, as is often the case under global change (Bonamour *et al.*, 2019).

Climate Change

Climate change describes long-term climatic alterations, which have been reported frequently in the history of earth and are considered independently from natural daily and annual cycles. There are indications that several factors have the capacity to cause changes in global climate, and often the combination of multiple factors leads to serious climatic effects (Crowley, 2000; Hegerl *et al.*, 2019). Solar variation has a direct impact on the earth's climate system and is subject to fluctuations. Solar luminosity shows an 11-year periodic change (Willson and Hudson, 1991). Additionally, long-term modulations over multiple solar magnetic activity cycles were described by Willson and Mordvinov (2003). On a larger scale, Milanković cycles, i.e. variations linked to the earth's orbit around the sun, also affect climatic changes and are notable for correlating with glacial periods, e.g. the major glaciation in the Northern Hemisphere around 2.75 million years ago (Willis *et al.*, 1999). Further natural mechanisms forcing climatic changes are volcanism, i.e. volcanic eruptions followed by large amounts of sulphur dioxide being injected into the stratosphere (Miles *et al.*, 2004; Cooper *et al.*, 2018), and plate tectonics, i.e. motion of tectonic plates affecting atmosphere-ocean circulation (Vérard and Veizer, 2019). Greenhouse gases are a major factor influencing the earth's climate and are emitted by natural mechanisms, e.g. volcanos (Stenchikov, 2016), and human activities (Kirk-Davidoff, 2018; Irving *et al.*, 2019). The emission of greenhouse gases, such as carbon dioxide, methane and nitrous oxide, leads to increasing temperatures because outgoing infrared light is trapped/reflected in the earth's atmosphere (Myhrvold and Caldeira, 2012; Kirk-Davidoff, 2018). Starting with the industrialization, anthropogenic influence on climate change became the dominant factor, mainly due to the usage of fossil fuels (Höök and Tang, 2013; Lelieveld *et al.*, 2019), and the scientific consensus on climate change is that changes from this time point on are mainly due to human activities (Chao and Feng, 2018) and are largely irreversible (Solomon *et al.*, 2009; Lickley *et al.*, 2019).

The ongoing anthropogenic climate change is known as a major threat to the survival of species and the integrity of ecosystems worldwide. Key factors of recent climate change are increasing temperature (Trenberth *et al.*, 2014; Cheng *et al.*, 2019) and ultraviolet radiation (UVR) (Bais *et al.*, 2019; Barnes *et al.*, 2019). Global warming, commonly referring to the mainly human-caused increase in global surface temperature, is the focus of countless studies and known to have serious effects on many physical and biological systems within terrestrial (Nolan *et al.*, 2018), marine (Pinsky *et al.*, 2019) and freshwater (Woodward *et al.*, 2010; Döll *et al.*, 2018) ecosystems. Changes in biologically effective UVR are also part of anthropogenic climate change with largely unknown consequences for the biosphere.

Ultraviolet radiation

Increasing ultraviolet radiation is, next to global warming, a major factor in terms of climate change and has strong effects on organisms regarding all levels of organization, affecting cells, tissues, organs, organ systems and whole organisms (reviewed in Kumar and Häder, 1999). The electromagnetic spectrum includes γ-radiation, X-rays, ultraviolet radiation, visible light, infrared radiation (heat), microwaves and radio waves. The UV ("beyond violet" from Latin ultra, "beyond") radiation represents a small part, ranging from 100 nm to 400 nm, which is below the range visible to the human eye (400 – 700 nm) (Diffey, 2002) and was discovered by Johann Wilhelm Ritter in the year 1801 (Frercks *et al.*, 2009). UVR is subdivided into three different spectral regions which were defined at the second international congress on light held in 1932 in Copenhagen (Coblentz, 1932): UVA (315 – 400 nm), UVB (280 – 315 nm) and UVC (100 – 280 nm). The exact spectral ranges are arbitrary and vary slightly depending on the discipline involved. Historically, UVB data refer to the integral between 280 – 315 nm or the integral between 280 – 320 nm (McKenzie *et al.*, 2004). Here, UVB refers to the often-used spectral range 280 – 320 nm because at 320 nm the solar spectrum "flattens out" and the biological action spectra approach a region of zero (Blumthaler and Webb, 2003). The amount of UVR reaching the earth's surface is mainly determined by the atmosphere, primarily by the ozone layer (Iqbal, 1983) resulting in 77 % of solar radiation being blocked before reaching the earth's surface (sun at zenith) with higher absorption in shorter wavelengths (Iqbal, 1983). UVC radiation, the most damaging light to biological systems, is blocked by the ozone layer and is ecologically irrelevant, as it does not reach the earth's surface (McKenzie *et al.*, 2003; Gouveia *et al.*, 2015). UVB is largely blocked by the ozone layer whereas UVA passes almost completely (Björn and McKenzie, 2015). Ozone (from greek "ozein" meaning "to smell") is a

gas with each molecule consisting of three oxygen atoms (O_3), which was discovered in 1840 by Christian Friedrich Schönbein (Schönbein, 1840). Approximately 10 % of the atmospheric ozone is located in the troposphere at a distance of 10 – 16 km and the remaining 90 % within the stratosphere at about 50 km distance from the earth's surface (Jacob, 2000). The term ozone layer refers to the large amount of ozone within the stratosphere. Ozone-depleting substances (ODSs), i.e. halogen source gases, including the most prominent substances chlorofluorocarbons (CFCs), participate in ozone destruction reactions, accumulate in the troposphere and reach the stratosphere by natural air motions (Jacob, 2000). The amount of atmospheric ozone shows steady lowering and additionally a larger decrease in spring at the polar regions (Piot and Glasow, 2009). Even though the emission of ozone-depleting chemicals, e.g. CFCs were reduced by the Montreal protocol since 1989 (Solomon, 2004), there are chemicals reducing the concentration of stratospheric ozone not included in this declaration (Ravishankara *et al.*, 2009; Laube *et al.*, 2014). Especially rising anthropogenic emissions of short-lived ozone-depleting chlorine substances, which are not considered by the Montreal protocol, could be important for the future climate (Hossaini *et al.*, 2015). Furthermore, changes in atmospheric circulation have led to a recent increase in stratospheric hydrogen chloride, also causing ozone-depletion (Mahieu *et al.*, 2014). The loss of stratospheric ozone results in changes of solar radiation reaching the earth. In addition to ozone effects, changes in aerosols, clouds and surface reflectivity have also contributed to UV changes during the last decades (Bais *et al.*, 2015).

Ultraviolet radiation in aquatic ecosystems

Aquatic ecosystems, i.e. freshwater and marine ecosystems, comprise the largest portion of the biosphere and are especially prone to changes in photic conditions. Particularly the exposure to UV radiation has a serious impact on aquatic organisms and is altered by interactions between climate change, ozone and UVR (Bais *et al.*, 2015; Bais *et al.*, 2019). UVR does not penetrate as deep into the water column as photosynthetically active radiation (PAR: 400 – 700 nm) but is still present in shallow waters. In marine ecosystems, UVB radiation has been detected at depths of 60 – 70 m and has been shown to inhibit biological processes even at depths between 20 – 30 m (Smith, 1989; Karentz *et al.*, 1994). UV transparency of aquatic ecosystems is highly variable and affected by multiple factors of climate change (Häder *et al.*, 2011; Williamson *et al.*, 2014). A major factor reducing the penetration depths of UV is the amount of dissolved and particulate organic carbon (DOC and POC) within the water column (Williamson *et al.*, 1996;

Piazena *et al.*, 2002; Williamson and Rose, 2010; Häder *et al.*, 2011) and, especially in freshwater ecosystems, the level of eutrophication (Bracchini *et al.*, 2004). Whereas models show that POC mainly affects absorption of UVA, DOC has been shown to have strong impact on UVB absorbance in the water column (Bracchini *et al.*, 2004). DOC degrades slowly in the water column but is readily fragmented into smaller subunits by solar UVR (Brinkmann *et al.*, 2003). These subunits are consumed by bacterioplankton (Klug, 2005) which, in turn, increases the UVB transparency (Pérez *et al.*, 2003). Additionally, increasing temperatures decrease dissolved organic material concentrations resulting in deeper UVB penetration (De Lange *et al.*, 2003).

An increase in global temperature is followed by decreasing sea-ice (Screen *et al.*, 2018), as well as decreased ice-cover in rivers and lakes in many regions (Fang and Stefan, 2009; Magee and Wu, 2017). Even thin layers of snow or ice significantly decrease the penetration of solar UV (Cockell and Córdoba-Jabonero, 2004) which makes temperature a serious factor that indirectly affects UV levels in aquatic ecosystems. Moreover, melting of the Arctic Ocean ice starts earlier and freezing later each year compared to the time before the warming (Kahru *et al.*, 2011). Additionally, the ice-cover duration of freshwater is affected, with much earlier spring melting (Butcher *et al.*, 2015) linked to negative impacts on aquatic ecosystems (Scheffer *et al.*, 1993) and changes in ambient UVR (Williamson *et al.*, 2014). Furthermore, higher water temperatures result in a thinner mixing layer as well as a longer growing season. Consequently, the exposure to UV radiation of organisms living in the upper layers of the water column is still increasing (Häder *et al.*, 2015).

UV exposure in fishes

Effects on skin

Although UVR represents only a small part of the solar spectrum, it has serious negative effects on organisms and changing photic conditions in aquatic ecosystems are often related to this wavelength band (Llabrés *et al.*, 2013; Peng *et al.*, 2017). Especially the higher energetic UVB range has a massive impact on aquatic ecosystems, particularly in shallow waters (Jokinen *et al.*, 2011). UVB has the potential to alter the structure of biologically relevant molecules, including proteins and deoxyribonucleic acid (DNA), and may lead to acute physiological stress (Sinha and Häder, 2002b; Dahms and Lee, 2010; McKenzie *et al.*, 2011) with the potential to affect whole ecosystems (Karentz *et al.*, 1991; Vincent and Roy, 1993; Sinha *et al.*, 1996).

The skin of fishes is especially prone to UV damage (Bullock, 1982) and several studies have shown direct negative effects of UVB on aquatic organisms (reviewed in Peng *et al.*, 2017). In fishes, exposure to elevated levels of UVB radiation has been shown to cause skin lesions, eye cataracts, dermal cancer and erythema (reviewed in Zagarese and Williamson, 2001). The eye of the rainbow trout (*Oncorhynchus mykiss*) exposed to an acute dose of UVB showed permanent lenticular and corneal damage (Cullen and Monteith-McMaster, 1993). Furthermore, cataractous changes of the eye including discrete anterior subcapsular opacities and peri-nuclear haze were also documented in the lenses of rainbow trout that received chronic UV exposure (Cullen *et al.*, 1994). Lahontan cutthroat trout (*Oncorhynchus clarki henshatvi*) show visible signs of sunburn upon exposure to simulated solar UVB radiation including a sloughing of the mucous cells, necrosis and edemas in the epidermis and dermis (Blazer *et al.*, 1997). In addition, cellular changes in the integument and characteristic of sunburn damage were observed in the sole (*Solea solea*) when exposed to elevated levels of UVB (McFadzen *et al.*, 2000). Moreover, in two cyprinids (*Phoxinus phoxinus* and *Chalcalburnus chalcoides*) and two salmonids (*Oncorhynchus mykiss* and *Salvelinus alpinus*) UVB radiation resulted in a reduced number of goblet cells (mucus secreting cells) in the dorsal epidermis (Kaweewat and Hofer, 1997). Some marine species react to enhanced UVB radiation by promoted production of mycosporine-like amino acids as UV screening compounds in the mucus, which partly absorb harmful UVR (Zamzow, 2004; Zamzow and Siebeck, 2006; Eckes *et al.*, 2008; Zamzow *et al.*, 2013).

Effects on immunity

Changes in the skin of fishes are often accompanied by infections, e.g. lesion-related contaminations, evoking immunological responses (Nowak, 1999) as shown for fungal infections in fishes exposed to excessive sunlight (Zagarese and Williamson, 2001). UVB radiation causes the loss of epidermal integrity and osmotic disturbances, facilitating the entry of pathogens (Zagarese and Williamson, 2001). Accordingly, extended periods of cloud-free days were followed by outbreaks of flexibacteriosis in cultured Atlantic salmon (*Salmo salar*) in Tasmania (Handlinger *et al.*, 1997). Generally, enhanced UVB radiation is known to have serious effects on immunological processes. Indian major carp (*Catla catla*), that were exposed to UVB radiation (145 µW/cm^2) for 15 minutes per day showed an increased immune response with higher glutamate oxaloacetate transaminase and glutamate pyruvate transaminase levels compared to individuals exposed to the same intensity of UVB for five or ten minutes daily (Sharma *et al.*, 2010). In the roach (*Rutilus rutilus*), a single dose of UVB (0.5 J/cm^2) was

followed by suppressed transiently mitogen-stimulated proliferation of blood lymphocytes, an altered functioning of the head kidney and blood phagocytes, induced granulocytosis and lymphocytopenia in the blood and increased plasma cortisol concentration (Salo *et al.*, 2000b). In roach, UVB radiation reduced lymphoproliferative responses and increased the proportion of granulocytes, whereas the proportion of lymphocytes in the peripheral blood decreased (Salo *et al.*, 2000a; Jokinen *et al.*, 2001). Common carp (*Cyprinus carpio*) showed modulated lymphocyte function (Markkula *et al.*, 2005) and an affected respiratory burst activity of blood and head kidney phagocytes, blood leukocyte counts and blood chemistry (Markkula *et al.*, 2009). Subramani *et al.* (2015) showed that Mozambique tilapia (*Oreochromis mossambicus*) exposed to enhanced UVB had suppressed primary and secondary antibody responses to a soluble protein antigen.

In summary, exposure to UVB irradiation alters the functioning of the innate and adaptive immune system in fishes and seriously affects the protection from a wide variety of microorganisms.

Effects on growth

Next to effects on the molecular and cellular level in fishes, effects of UVB radiation on digestive enzymes were shown in a fish species, the Indian major carp, with decreased enzyme activities resulting in improper digestion and poor growth (Sharma *et al.*, 2010). European whitefish larvae (*Coregonus lavaretus*) (Ylönen and Karjalainen, 2004), Atlantic Salmon (Jokinen *et al.*, 2008; Jokinen *et al.*, 2011) and the transitory fish *Girella laevifrons* (Pulgar *et al.*, 2017) also showed reduced growth following enhanced UVB radiation. Next to impaired digestion (Sharma *et al.*, 2010), costly repair mechanisms are often the consequence of the described harms, e.g. DNA damage, caused by UVB and might result in impaired growth. Olson and Mitchell (2006) showed that salmonid larvae (brook trout, rainbow trout) rely solely on energetically expensive nucleotide excision repair and suggest that impaired growth may be connected to those costs. Furthermore, in European whitefish, it has been shown that UVB radiation suppresses energy allocation to digestion (Ylönen *et al.*, 2004). In addition, plasma cortisol was shown to be elevated after exposure to UVB (Salo *et al.*, 2000a; Markkula *et al.*, 2007) and an increased cortisol level changes feeding behavior, carbohydrate metabolism and growth of fish (de Oliveira Fernandes and Volpato, 1993; Gregory and Wood, 1999). To sum up, alterations in metabolism caused by enhanced ambient UVB radiation were shown to have the potential to impair growth in fishes. Accordingly, it was shown that UVB radiation directly

affects oxygen consumption, increased swimming activity and restless behavior in juvenile rainbow trout (Alemanni *et al.*, 2003).

Effects on behavior

UV effects at the behavioral level, e.g. the above-mentioned effects on activity (Alemanni *et al.*, 2003), have been observed in multiple contexts and various fish species. Juvenile black sea bream (*Acanthopagrus schlegeli*) showed UVB avoidance behavior in a tank irradiated by a UVB lamp with half of the tank covered by UV-blocking film (Fukunishi *et al.*, 2006). UVB-avoidance was also found in red sea bream larvae (*Pagrus major*), which showed shade-seeking behavior in the presence of UVB (Sharma *et al.*, 2007). Atlantic cod larvae (*Gadus morhua*) showed lower prey consumption after exposure to UVB radiation (Fukunishi *et al.*, 2013a). Moreover, the escape performance of Atlantic cod was significantly lower after exposure to enhanced UVB than in the control group in the presence of two-spotted goby (*Gobiusculus flavescens*) as a predator (Fukunishi *et al.*, 2012).

Behavioral responses can be classified into changes based on physical stress, mediated e.g. by higher cortisol levels, or on visual perception of UV light, e.g. avoidance. Visual perception of UVR has been shown for several fish species (reviewed in Losey *et al.*, 1999). However, visual UV sensitivity only refers to UVA radiation and can change over the course of a lifetime (reviewed in Bowmaker, 1990). UVB vision in fishes has not been shown yet.

Effects on oxidative stress

It can be assumed that the harmful consequences of UVR on many biological processes are mediated by the formation of reactive oxygen species (ROS) followed by oxidative stress (Häder *et al.*, 2007). ROS are a family of oxygen-centered species formed as by-products of normal metabolic processes in cells, including superoxide anion, hydroxyl radicals, singlet oxygen and hydrogen peroxide (Birben *et al.*, 2012). ROS are excessively produced after exposure to ionizing and non-ionizing radiation and/or redox cycling chemicals (Zhang *et al.*, 1997) accompanied by cell injuries (Peak and Peak, 1989; Gajewski *et al.*, 1990). Numerous molecules in cells, e.g. aromatic amino acids, absorb UVR and the excitation energy has the potential to form various species of ROS resulting in a production of extremely reactive hydroxide ions (Banaszak and Lesser, 2009). The toxic effects of ROS on organisms, ranging from damage on DNA, enzymes, membrane proteins and lipids as well as photosystem components, are commonly described as photooxidative stress (Lesser, 2006).

Antioxidant defenses antagonize oxidative stress and can be categorized into enzymatic and nonenzymatic antioxidants (Lesser, 2006). Antioxidant enzymes include superoxide dismutase (SOD), catalase and ascorbate or glutathione peroxidases, which have the potential to scavenge or quench ROS by breaking down and removing free radicals (Lesser *et al.*, 2001; Banaszak and Lesser, 2009). In a multi-step process, in presence of cofactors (e.g. copper, zinc, manganese and iron), dangerous oxidative products are converted to hydrogen peroxide and then to water (Nimse and Pal, 2015). Nonenzymatic antioxidants include ascorbic acid (i.e. vitamin C), glutathione, tocopherols, carotenoids, small-molecule antioxidants (i.e. uric acid) and compatible solutes (e.g. mannitol, dimethylsulfide, dimethylsulphoniopropionate and the UVR-absorbing mycosporine-like amino acids) (Lesser, 2006). These nonenzymatic antioxidants interrupt free radical chain reactions (Nimse and Pal, 2015).

Carotenoids are pigments which play a major role in the protection against photooxidative processes and are efficient antioxidants scavenging singlet molecular oxygen and peroxyl radicals generated during photooxidation (Stahl and Sies, 2002). The protection of biological systems against ROS by carotenoids mainly depends on physical quenching during which the energy of the excited oxygen is transferred to the carotenoid molecule (Truscott, 1990). Rotational and vibrational interactions between the excited carotenoid and the surrounding solvent lead to dissipation of the energy (Stahl and Sies, 2003). During the process of physical quenching, the carotenoid molecule is not destroyed and may undergo further cycles (Stahl and Sies, 2002). Chemical quenching, involving carotenoid oxidation, is responsible for the final decomposition of carotenoids called bleaching (Stahl and Sies, 2002). Carotenoids represent exceptional antioxidants as they are involved in a variety of other physiological processes, e.g. immunity and expression of color signals (Blount, 2004; Pérez-Rodríguez, 2009; Pike *et al.*, 2010; Toomey *et al.*, 2010).

The three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*), which was used as model organism in this thesis, is a small (5 – 8 cm) teleost fish species which belongs to the family of Gasterosteidae (Mattern and McLennan, 2004), and is distributed across the 35° and 70° degree of latitude in the Northern Hemisphere (Wootton, 1976). Presumably originating from seawater, three-spined sticklebacks have colonized freshwater habitats (Bell and Foster, 1994) and can nowadays be found in seawater, brackish- and freshwater where they inhabit shallow lentic and flowing aquatic habitats (Bell and Foster, 1994). Based on their migration behavior,

populations can be divided into resident freshwater and anadromous populations. Whereas resident populations spend their whole life in freshwater, anadromous populations reproduce in freshwater habitats and migrate downstream to the sea after the breeding season in summer (Wootton, 1984; FitzGerald and Wootton, 1993).

Individuals used in the first chapter of this study were wild caught and obtained from a shallow freshwater pond near Euskirchen, Germany (50°38'N, 6°47'E). In *chapter II*, wild caught sticklebacks from an anadromous population were used, which were caught during spring migration on the island of Texel, the Netherlands. Study animals used in *chapters III* and *IV* were laboratory-bred descendants from three-spined sticklebacks obtained from the anadromous population used in *chapter II*. Characteristic for this species are three moveable spines on the dorsal body side as well as two ventral spines instead of pelvic fins (Wootton, 1984). In contrast to most teleost fish species, sticklebacks have neither cycloid nor ctenoid scales but developed lateral bony plates (Roberts, 1993) in four different morphs (Banbura and Bakker, 1995).

The three-spined stickleback represents an ideal model organism which is widespread throughout various lighting habitats and easy to breed and keep in the laboratory (Bell and Foster, 1994; Rowe *et al.*, 2004). Starting with mainly behavioral studies (reviewed in Huntingford and Ruiz-Gomez, 2009), the number of fields that use this fish as a model organism has risen incredibly (Norton and Gutiérrez, 2019) and sticklebacks are now firmly established as a biological 'supermodel' (Gibson, 2005).

Three-spined sticklebacks and UV

Three-spined sticklebacks inhabit shallow waters (Wootton, 1984) where they are naturally exposed to ambient UVR. Next to absorption maxima within the PAR range of the solar spectrum at 452 nm (blue), 529 nm (green) and 604 nm (red) (Lythgoe, 1979), there is a further cone class located in the retina of three-spined sticklebacks which absorbs wavelengths in the UVA range with an absorption maximum located at 360 nm (Lythgoe, 1979). Furthermore, sticklebacks reflect in the UV spectral range in different body regions (Rick *et al.*, 2004; Rowe *et al.*, 2004) and use UVA during intraspecific communication which has been shown in different contexts, e.g. shoaling (Modarressie *et al.*, 2006; Hiermes *et al.*, 2015c), territorial aggression (Rick and Bakker, 2008b) and mate choice (Rick *et al.*, 2006; Rick and Bakker, 2008c). Using UV signals for communication is beneficial as it enables the transmittance of information invisible to a variety of predators, e.g. adult rainbow trout or perch (Bowmaker, 1990). Additionally, because UV is strongly scattered, such signals allow short-distance

communication without attracting predators (Losey *et al.*, 1999). Thus, Cummings *et al.* (2003) described such intrasexual visual communication using UV signals as 'private ultraviolet channel'. Next to intraspecific communication, sticklebacks use UV-perception during foraging (Rick *et al.*, 2012) and habitat selection (Rick and Bakker, 2010).

In shallow waters, UVA and UVB appear together and when using UV signals for communication individuals are not only exposed to UVA, but also to higher energetic harmful UVB, which is not visible to sticklebacks. Furthermore, next to beneficial aspects of UVA additional detrimental effects of UVA have been observed on male breeding coloration and sperm velocity in this species (Rick *et al.*, 2014). Knowledge on the impact of UVB on the biology of three-spined sticklebacks is scarce and is the focus of the present thesis.

Three-spined sticklebacks' reproduction

The reproductive phase of sticklebacks generally starts in spring with variations concerning ecotypes and latitudes (Ishikawa and Kitano, 2020). At the beginning of the reproductive phase, most populations inhabit the littoral zone of freshwater habitats where males separate from shoals and occupy territories in shallow waters (Wootton, 1976). When becoming reproductively active, males express a characteristic red coloration at the throat and belly region as well as a blue coloration of the iris (Fig. 1; Wootton, 1976). The kidney of male sticklebacks produces a glycoprotein, spiggin, whose secretion is controlled by androgenic hormones (Jakobsson *et al.*, 1999) and males use it as a 'glue' to build a tunnel-shaped nest out of algae and other vegetation (Fig. 1). During courtship, the male performs a characteristic 'zig-zag-dance' to attract a gravid female and court it to the entrance of the nest (Wootton, 1976). After a female has spawned in the nest, the male fertilizes the eggs and expels the female from its territory (Wootton, 1976). Males can collect eggs from different females in a period of one to ten days and afterwards enter the parental phase (Kraak *et al.*, 1999). Depending on the temperature, the offspring hatches after five to 20 days (Wootton, 1984). During brood care, the male defends eggs and offspring against potential predators, provides oxygenated water by fanning with its pelvic fins and removes unfertilized eggs and dead or diseased embryos (Wootton, 1976).

In addition to the described reproductive behavior, alternative mating strategies have evolved in three-spined sticklebacks. A widespread alternative method is the so-called sneaking behavior, in which male sticklebacks steal fertilizations by fertilizing eggs within the nests of other males (e.g. Largiadèr *et al.*, 2001; Bakker *et al.*, 2006).

Fig. 1: This photograph shows a male and a female three-spined stickleback during reproduction. The male (top, left) shows the typical nuptial coloration consisting of a red throat and abdomen together with a blue iris. While the female is spawning within the tunnel shaped nest (bottom, center), the male guards its territory.

Pre-mating traits in male three-spined sticklebacks

Physical body traits can affect mating preferences in three-spined sticklebacks as shown for strong effects of body size in terms of assortative mating (Nagel and Schluter, 1998), which may significantly reduce hybridization levels between populations (Bolnick and Kirkpatrick, 2015).

However, the most prominent pre-mating trait in sticklebacks is the expression of a characteristic nuptial coloration, i.e. the red coloration of throat and belly in male individuals, which is a famous and well-studied example of a color signal in nature (reviewed in Rowland, 1994). It was also shown that UV signals contain important information for visual mate choice in sticklebacks and a removal of UV light makes stickleback males appear less attractive to females (Rick and Bakker, 2008a). Sexual ornamentation in three-spined sticklebacks plays an important role in male-male competition (Bakker, 1986) as well as during female mate choice (e.g. McLennan and McPhail, 1989; Bakker and Milinski, 1993). Additionally, the red coloration provides a strong contrast to the iridescent blue eyes which has been shown to play a role in female mate choice (Flamarique *et al.*, 2013). The red nuptial coloration is based on carotenoid pigments, mainly astaxanthin and lutein (Wedekind *et al.*, 1998), which are synthesized by bacteria, plants, fungi and algae (Olson and Owens, 1998). Therefore, sticklebacks have to obtain carotenoids from their diet and deposit them in the integument

(Wedekind *et al.*, 1998; McGraw and Hill, 2006). Intense coloration requires the intake of food containing high-quality carotenoids and, therefore, is linked to higher costs in terms of an increased foraging effort (e.g. Endler, 1983). Next to their role as color signals, carotenoids are involved in many physiological processes. As described above, they have the capacity to reduce oxidative stress and are known to be a potent immunostimulant (McGraw and Ardia, 2003). Consequently, carotenoids represent a potential key resource for allocation trade-offs between physiological processes, e.g. expression of color signals, immunity and oxidative stress defense (reviewed in Blount, 2004). Moreover, allocation of carotenoids can result in trade-offs between pre-mating secondary sexual traits and post-mating ejaculate quality, especially when oxidative stress is high (Mehlis *et al.*, 2015; Simmons *et al.*, 2017; Koch and Hill, 2018).

The investment in the pre-mating sexually selected color traits in the present thesis was determined by conducting spectrophotometric reflectance measurements, which allowed an objective quantification of the expression of the nuptial coloration in reproductive active stickleback males. Furthermore, reflectance data were used to conduct visual modelling which allows an estimation of how the visual signals would be perceived by a conspecific (Stoddard and Prum, 2008). For methodological details, see *chapters II* and *III*.

In sticklebacks, extended phenotypes may play a role in signaling as well (Barber *et al.*, 2001). Next to the well-documented nest-building behavior (Wootton, 1976; Rowland, 1994), several studies examined nests as extended phenotypes (Barber *et al.*, 2001; Östlund-Nilsson and Holmlund, 2003; Rushbrook *et al.*, 2008; Head *et al.*, 2017) and stickleback nests were shown to provide information regarding male quality (Barber *et al.*, 2001).

Taken together, males can signal their quality through physical traits, e.g. size or the expression of color signals as well as extended phenotypes, e.g. nest traits. However, the key determinant of female mate choice decisions is the expression of carotenoid-based nuptial coloration (e.g. Bakker, 1990; Bakker and Milinski, 1993; Bakker and Mundwiler, 1994) reflecting males' quality, e.g. in terms of parasite resistance (Milinski and Bakker, 1990), which also plays a key role in intrasexual selection (e.g. Rowland, 1989; Bakker and Milinski, 1993; Bakker, 1994). Consequently, effects on pre-mating sexually selected traits directly affect the fitness of sticklebacks, which makes them an appropriate measure to examine the effect of enhanced ambient UVB radiation, representing a serious environmental stressor, on the fitness of *G. aculeatus*.

Post-mating traits in male three-spined sticklebacks

Reproductive success is, next to mate acquisition (pre-mating), mainly influenced by fertilization success (e.g. Andersson, 1994). In sexual selection of *G. aculeatus,* post-mating sexual traits are particularly relevant because stealing fertilizations (sneaking) is a common tactic of male sticklebacks resulting in a high risk of sperm competition (Largiadèr *et al.*, 2001). The amount of sperm during the reproductive phase of sticklebacks is limited as spermatogenesis mainly takes place in the time span from late autumn to early winter preceding the breeding season (Borg, 1982). However, Sokolowska and Kulczykowska (2006) examined that spermatogenesis is not completely stopped during spring and summer, at least for two different populations. Nevertheless, sperm supply can be regarded as limited during the breeding season, which is supported by higher numbers of sperm present in virgin males than in individuals that have mated multiple times (Zbinden *et al.*, 2001). Sperm number and mass of the ventrolaterally located testes are significantly positively correlated (Zbinden *et al.*, 2001) and can both be seen as a measure to evaluate reproductive investment, which was used in *chapters II* and *III* of this thesis. In addition, faster and/or more motile sperm were shown to affect the fertilization success in externally fertilizing species (Pizzari and Parker, 2009) and, thus, next to the amount of sperm, sperm movement is an important factor during fertilization.

Stressful conditions are known to affect sticklebacks' reproductive performance and sperm movement was shown to be affected by environmental changes, e.g. temperature (Mehlis and Bakker, 2014) or UVA radiation (Rick *et al.*, 2014). In *chapters II* and *III* of the present thesis, I examined the influence of enhanced ambient UVB radiation on post-mating, sperm-related traits during the reproductive phase (*chapter II*) and from an early life stage on, during the major growth phase (*chapter III*). Testis masses were measured and the number of sperm microscopically determined by pestling testes and counting sperm numbers using a Leja counting chamber. Sperm suspensions together with artificial "ovarian fluid" (Elofsson *et al.*, 2006) were also used to examine sperm movement by applying computer-assisted sperm analyses (Kime *et al.*, 2001). In *chapter II*, the sperm suspension was also used to assess a possible link between sperm morphology and movement (Humphries *et al.*, 2008) by measuring sperm head length, mid-piece length and tail length as well as the head-to-tail length ratio (HT-ratio = (head length + midpiece length) / tail length). Methodological details are given in *chapters II* and *III*. Effects on swimming performance can be assumed to directly affect an individual's fertilization success as higher velocity in sperm likely enhances the probability of finding unfertilized eggs (Pizzari and Parker, 2009; Kowalski and Cejko, 2019).

In summary, post-mating sperm traits, i.e. sperm quantity and quality, represent a suitable measure to determine the impact of the environmental stressor 'enhanced ambient UVB radiation' on an individual's direct fitness. Especially in externally fertilizing species with a high risk of sperm competition, consequences of detrimental effects on sperm quantity and/or quality are highly relevant for reproductive success and therefore represent a major aspect of this thesis.

Trade-offs between pre- and post-mating traits

When resources are limited and/or stress is prevalent, trade-offs may occur between mate-acquisition (pre-mating) or fertilization (post-mating), which was e.g. shown for carotenoid-based sexual ornamentation and sperm resistance to oxidative stress in zebra finches (*Taeniopygia guttata*) (Tomášek *et al.*, 2017). Thus, studying the impact of an environmental stressor on reproductive performance requires the consideration of both pre- and post-mating traits and particularly the relationship between both. Resource allocation directed to pre-mating traits at the expense of post-mating traits may ensure the acquisition of mating-partners by enhancing the competitiveness against rivals, which is essential for sexual reproduction. However, if the investment in pre-mating traits is at the disadvantage of post-mating traits, this may result in reduced fertilization success and consequently reduce the individual's direct fitness.

The phenotype-linked fertility hypothesis (PLFH) by Sheldon (1994) suggests that the honesty of sexual ornaments is maintained through a link with fertility. The occurrence of potential trade-offs between ornaments (pre-mating) and fertility (post-mating) would result in a rejection of the PFLH as the honesty of ornaments could not be preserved. Resource allocation towards pre-mating traits, i.e. maintaining sexual attractiveness, at the expense of post-mating traits would mean that potential mating partners could not assess the quality, e.g. fertility, based on the expression of ornaments. Such trade-offs are predicted by sperm competition theory (SCT) (Parker, 1990), which predicts a trade-off between the investment in pre- and post-mating traits under resource limitation. Accordingly, Mehlis *et al.* (2015) found a positive relationship between pre- and post-mating traits in absence of food limitation, but a negative relationship under resource limitation. Whether the environmental stressor UVB can impact the investment in pre- and/or post-mating traits in three-spined sticklebacks is investigated in *chapter II* and *chapter III*.

Aim of this thesis

The present thesis aims to examine the effects of a changing photic environment in terms of enhanced ambient UVB radiation on physiological processes, resource allocation and behavioral aspects in three-spined sticklebacks. All chapters of my thesis are based on an experimental approach, which included a manipulated natural solar spectrum in an outdoor-setup (Fig. 2). I assigned the experimental animals to two different exposure treatments differing solely in the amount of UVB radiation. To create different lightning conditions, I manipulated the UVB part of the solar spectrum alone by using artificial lighting in addition to the natural sunlight whereas in the control treatment individuals received unmanipulated solar radiation.

In *chapter I*, I investigated the consequences of exposure to enhanced ambient UVB radiation on immunological processes. The immunity of fishes is known to be affected by abiotic stressors which has already been studied, e.g. regarding temperature (e.g. Li *et al.*, 2019; Ignatz *et al.*, 2020) and ultraviolet radiation (e.g. Ceccato *et al.*, 2016; Lawrence *et al.*, 2020). Whereas various studies on the effects of UVR used single doses (summarized in Lawrence *et al.*, 2020), I experimentally manipulated the photic environment under semi-natural conditions and used chronic UVB exposure to reveal the immunological, physical and behavioral consequences of enhanced but ecologically relevant UVB radiation. Thus, *chapter I* addresses the immunomodulatory effects of chronic UVB exposure on three-spined sticklebacks. The major immune organ in fishes, the head kidney, was examined to reveal the investment in the innate and adaptive immunity of individuals being stressed by enhanced UVB, compared to individuals exposed to solar radiation. Therefore, I determined the relation of granulocytes (innate immunity) and lymphocytes (adaptive immunity) obtained from the head kidney. Moreover, I calculated the relative mass of the spleen, a lymphoid organ involved in adaptive immunity, and the change in body condition compared between individuals exposed to enhanced UVB and the control group.

In *chapter II* and *chapter III*, I investigated the effects of enhanced ambient UVB on sexually selected pre- and post-mating traits in male sticklebacks. At the pre-mating level, I focused on sexual ornamentation (*chapters II* and *chapter III*) and nests as extended phenotype (*chapter III*). At the post-mating level, the focus was on sperm morphology (*chapter II*) as well as sperm number and movement (*chapters II and III*). In *chapter II*, I investigated the effects of enhanced ambient UVB on adult, reproductively active males. Here, I explored the consequences of environmental stress for already developed pre- and post-mating fitness-

relevant traits. Whereas individuals may attenuate harmful effects on fitness-relevant traits by shifted resource allocation preceding the expression of pre- and post-mating traits, *chapter II* deals with the direct impact of UVB stress during the reproductive phase. In *chapter III*, sticklebacks were exposed to enhanced UVB from the juvenile stage on, during the major growth phase. By using such long-term exposure, I examined the effects of developmental exposure to enhanced, but ecologically relevant, ambient UVB levels on growth, pre-mating and post-mating sexual selection as well as their interrelationships. In addition, testicular antioxidant capacity and testes as well as skin melanization were quantified to reveal potential counter-adaptations to persistent UVB stress or to identify possible mechanisms underlying the expression of post-mating traits. This central part of the present thesis deals with the investment in pre- and post-mating traits when being exposed to a stressful environment and especially with trade-offs regarding resource allocation towards pre- or post-mating traits which can directly affect an individual's fitness.

The final part of my thesis, *chapter IV*, considers the behavioral consequences of long-term exposure to ecologically relevant UVB levels, particularly with regard to predator-prey behavior. Here, I investigated predator–prey interactions which directly affect an individual's survival and, thus, showed that environmental stress can affect crucial behavioral processes.

Fig. 2: Experimental set-up used for creating the two exposure treatments and animal husbandry which was used in all experiments of the present thesis. Four circular outdoor tanks (2500 l, diameter of 2 m) were arranged next to each other in a square of 36 m². Within each tank, twelve enclosures were mounted serving as holding tanks for three-spined sticklebacks which were used as experimental animals. Two exposure treatments were generated by mounting a UVB lamp above every second enclosure, whereas the remaining enclosures were equipped with a dummy, providing similar shading as in enclosures with UVB lamps.

Author's contributions

Chapter I

Simon Vitt, Anna K. Rahn, Theo C. M. Bakker and Ingolf P. Rick designed the study. Simon Vitt, Lisa Drolshagen, and Anny K. Rahn collected the data. Simon Vitt, Lisa Drolshagen, Anna K. Rahn and Ingolf P. Rick did the statistical analyses. Simon Vitt wrote the manuscript, supported by Theo C. M. Bakker and Ingolf P. Rick. Jörn P. Scharsack reviewed the final manuscript providing important feedback.

Chapter II

Simon Vitt, Theo C. M. Bakker and Ingolf P. Rick designed the study. Simon Vitt and Marion Mehlis-Rick collected the data. Simon Vitt and Ingolf P. Rick did the statistical analyses. Simon Vitt wrote the manuscript supported by Marion Mehlis-Rick, Theo C. M. Bakker and Ingolf P. Rick.

Chapter III

Simon Vitt, Theo C. M. Bakker and Ingolf P. Rick designed the study. Simon Vitt collected the data. Simon Vitt and Ingolf P. Rick did the statistical analyses. Simon Vitt wrote the manuscript supported by Theo C. M. Bakker and Ingolf P. Rick.

Chapter IV

Simon Vitt, Janina E. Zierul, Theo C. M. Bakker and Ingolf P. Rick designed the study. Simon Vitt and Janina E. Zierul collected the data. Simon Vitt, Janina E. Zierul and Ingolf P. Rick did the statistical analyses. Simon Vitt wrote the manuscript supported by Theo C. M. Bakker, Janina E. Zierul and Ingolf P. Rick.

This thesis includes four single manuscripts, i.e. *chapters I–IV*, which have been published in different scientific journals. Consequently, each manuscript must be comprehensive in itself which makes recurrent descriptions inevitable. Format and layout of the following manuscripts were adapted to fit the layout of this thesis, but the content has not been changed. All references are listed at the end of this thesis.

Chapter I

Enhanced ambient UVB light affects growth, body condition
and the investment in innate and adaptive immunity in
three-spined sticklebacks (*Gasterosteus aculeatus*)

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Note: This chapter is written in British English according to the published version.

Chapter I

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and Ingolf P. Rick

Abstract

With ongoing environmental change, ultraviolet-B radiation (UVB) reaching the Earth's surface has increased over recent decades with consequences for terrestrial and also aquatic ecosystems. Despite evidence for direct physiological and immunological responses of aquatic animals following enhanced UVB exposure, studies investigating indirect impacts of ambient UVB radiation are scarce and mainly used only single doses and/or artificially high amounts of UVB. In the present study, the influence of chronic exposure to elevated UVB levels on growth, body condition and immune function was investigated in three-spined sticklebacks (*Gasterosteus aculeatus*). Fish were kept outdoors for 68 ± 2 days under two different spectral conditions; one group was exposed to natural solar radiation (UVB-normal), while the other group received additional UVB light for four hours daily (UVB-enhanced). Enhanced UVB radiation was within the range of UVB levels measured at the study site. Fish length and weight were determined at the beginning and end of the experiment to compare growth and body condition between the two treatment groups. At the end of the experiment, the splenosomatic index and the granulocyte-to-lymphocyte ratio were determined as immune parameters. Fish from the UVB-enhanced group showed a reduced growth and body condition as well as a lower splenosomatic index compared to the UVB-normal group. Furthermore, UVB-treated fish had a higher granulocyte-to-lymphocyte ratio representing a relatively higher activation of innate compared to adaptive immunity. Consequently, increased but ecologically relevant levels of ambient UVB negatively affect growth and body condition and have a considerable impact on immunity in three-spined sticklebacks.

Introduction

Solar ultraviolet radiation, especially in the UVB wavelength range (280–320 nm), has significant effects on terrestrial and aquatic organisms at all levels of biological organisation and plays an important role in the context of environmental change (Häder *et al.*, 2007; Williamson and Rose, 2010; Williamson *et al.*, 2014). Ozone depletion leads to an increase in the amount of solar UVB reaching Earth's surface (Stuart *et al.*, 2004) affecting mainly polar regions, but also the mid-latitudes of the northern and southern hemispheres (Reuder *et al.*, 2001). In aquatic ecosystems, the UVA-wavelength range (320-400 nm) represents the main part of solar UV radiation as it penetrates deeper into the water column than UVB, but UVB is more energetic and therefore plays a role as stressor for aquatic organisms especially in shallow habitats (e.g. Bothwell *et al.*, 1994; Bancroft *et al.*, 2007; Sucré *et al.*, 2011).

Impacts of UVB radiation include detrimental effects on various levels of animal organisation. On the molecular level, photo-induced damage to macromolecules can be related to UVB in terms of DNA and RNA mutations as well as damage to enzymes and membranes (Häder *et al.*, 1998; Dahms and Lee, 2010). Furthermore, direct effects of UV radiation (UVR) on fatty acids in the skin, ocular tissue and on dorsal muscle as well as growth were found in Atlantic salmon (*Salmo salar*) (Arts *et al.*, 2010). UV-induced genetic alterations may lead to negative impacts on the ontogenetic development (Sinha and Häder, 2002a). Exposure to UVB can further lead to modifications of osmoregulatory functions as was found for the larval stage of the European seabass (*Dicentrarchus labrax*) in terms of lower numbers of integumentary ionocytes and mucous cells. Moreover, UVB is known to negatively affect growth and body condition (Jokinen *et al.*, 2008; Jokinen *et al.*, 2011) and can lead to an increased mortality as shown for eggs of the Atlantic cod (*Gadus morhua*) (Kouwenberg *et al.*, 1999) and embryos of other fishes (Llabrés *et al.*, 2013). UVB can also interact with other environmental factors such as thermal conditions (Carreja *et al.*, 2016; Seebacher *et al.*, 2016), and it can, for example, increase the susceptibility to parasitic infections in fish (Cramp *et al.*, 2014). Increases in UVR can have substantial effects on trophic-level interactions as well, even though the detailed relationships are far from being understood (but see Bothwell *et al.*, 1994; Häder *et al.*, 2007).

A number of studies have also demonstrated immunomodulatory effects of UVB radiation. The immune system of teleost fishes generally consists of non-specific innate and highly specific adaptive or acquired immunity components (Magnadóttir 2006; Uribe et al. 2011). On the cellular level, immediate, but rather unspecific responses are carried out by cells of the innate immune system, such as granulocytes, whereas cells of the adaptive immune

system, such as B and T lymphocytes, mediate highly specific immune responses and long-lasting immune memory (Flajnik and Kasahara, 2010).

UVR-induced immunosuppression has been shown in fishes (e.g. Subramani *et al.*, 2015), insects (e.g. Debecker *et al.*, 2015), amphibians (e.g. Ceccato *et al.*, 2016), birds (e.g. Blount and Pike, 2012), mammals (e.g. Uberoi *et al.*, 2016) and humans (reviewed in Ullrich, 2016). In fish, Salo et al. (1998) studied the effects of a single dose of UVB on the non-specific immune system of the roach (*Rutilus rutilus*) with regard to random and directed migration of granulocytes. Spontaneous cytotoxicity of granulocytes towards target cells was suppressed one day after irradiation and even at 14 days post-irradiation (Salo *et al.*, 1998). Further photo-immunological studies showed an increased proportion of granulocytes and decreased proportion of lymphocytes in the peripheral blood of roach, carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) in response to a single dose of UVB (Salo *et al.*, 2000a; Salo *et al.*, 2000b). Additionally, decreased lymphoproliferative responses were observed in roach after short-term exposure to UVB (Jokinen *et al.*, 2001). In a long-term experiment using juvenile Atlantic salmon (*Salmo salar*), enhanced UVB reduced haematocrit value and plasma protein concentrations and affected plasma immunoglobulin concentrations (Jokinen *et al.*, 2008). Most studies on fish have explored the effects on embryos and larval stages using a single dose of UVB or short exposure times of only a few days (but see Markkula *et al.*, 2005; Fukunishi *et al.*, 2013b). Furthermore, artificial conditions were used such as restriction of motion during exposure concomitant with additional stress through handling of the experimental fish (e.g. Markkula *et al.*, 2005; Markkula *et al.*, 2006). Moreover, studies on long-term effects exposed fish to UVB radiation several times a week but did not use a chronic UVB exposure by daily application (Markkula *et al.*, 2009).

In the present study, three-spined sticklebacks (*Gasterosteus aculeatus*) were chronically exposed to either elevated, but naturally occurring levels of UVB radiation (UVB-enhanced) or natural daylight conditions as control treatment (UVB-normal) on a daily basis for several weeks. Three-spined sticklebacks inhabit shoreline areas of marine, brackish and freshwater habitats in the northern hemisphere (Wootton, 1984), with shallow waters being characterised by elevated levels of UVR. Sticklebacks are capable of perceiving light in the UVA range (Rowe *et al.*, 2004) and UV wavelengths are used in this species for intraspecific communication (e.g. Modarressie *et al.*, 2006; Rick *et al.*, 2006; Rick and Bakker, 2008b; Hiermes *et al.*, 2015c), visual foraging (Rick *et al.*, 2012) and habitat selection (Rick and Bakker, 2010). Moreover, increased levels of ambient UVA light during the nesting period had

negative effects on sperm quality and sexual ornamentation in stickleback males (Rick *et al.*, 2014).

Here, the effects of a long-term UVB exposure on growth, body condition, the relative spleen mass and immune functioning were quantified and compared between the two treatment groups (UVB-enhanced, UVB-normal). Our expectation was that chronic exposure to enhanced UVB radiation leads to a shifted immune activation, resulting in a higher ratio of granulocytes to lymphocytes due to UVB-induced non-specific inflammatory processes.

Materials and methods

Study animals

About 320 juvenile three-spined sticklebacks were collected in November and December 2012 from a shallow freshwater pond near Euskirchen, Germany (50°38'N, 6°47'E) with the permission of the local forestry department. At the Institute for Evolutionary Biology and Ecology, University of Bonn, fish were maintained in one outside stock tank (volume 700 l; temperature 8 ± 1 °C with a tap-water flow rate of $3 \, l \, min^{-1}$ and air ventilation) for three months prior to the start of the experiments. Fish were fed *ad libitum* three times a week with defrosted chironomid larvae. To rule out a possible influence of UVB on ectoparasites (e.g. *Gyrodactylus* sp.), which may have an indirect effect on fish immunity, all sticklebacks were treated with an anthelmintic agent (Gyrodol 2, JBL, Neuhofen, Germany). The absence of ectoparasites was confirmed by microscopy before the start of the experiments.

The study conforms to the Association for the Study of Animal Behaviour Guidelines for the use of animals in research as well as to the legal requirements of Germany. No further licences were needed.

Experimental set-up

At the beginning of the experimental phase, on 16 January 2013, 144 subadult fish with an average standard length of 3.362 ($\pm$ SE 0.02 cm) and a weight of 0.461 ($\pm$ SE 0.01 g) were transferred from the outside stock tank to four circular outdoor tanks with a volume of 2500 l each and a diameter of 2 m (AquaTech, Kitzbühel, Austria), arranged in a square of 36 m² with uniform sun exposure. The tanks were equipped with a filter (PonDuett 3000, Pontec, Hörstel, Germany) and six water-permeable enclosures (39 × 28 × 28 cm), which were dipped 20 cm deep into the water column. UVB lamps (G8T5E, 8W, Sankyo Denki, Kanagawa, Japan) were installed 10 cm above the water surface of every second enclosure to create lighting conditions consisting of natural daylight and artificially enhanced UVB radiation (UVB-enhanced). The

remaining enclosures were illuminated by natural daylight (UVB-normal). In this case, a dummy (grey PVC, 2 × 40 cm) was installed above the enclosures, providing the same shading as the UVB lamps used in the UVB-enhanced treatment. Twelve days after transferring the test fish into the outdoor tanks UVB lamps were switched on for four hours daily around noon (11:00 a.m. – 03:00 p.m.). Fish were kept in equal-sized, mixed-sex groups of six individuals per enclosure for nine weeks resulting in 144 individuals (24 groups); half of them were assigned to UVB-enhanced and half to UVB-normal conditions. Sticklebacks in each enclosure were marked individually by clipping the tip of the dorsal spines in various combinations to enable the determination of change in growth and body condition for each individual. During the whole experimental phase, fish were fed three times a week *ad libitum* with defrosted chironomid larvae, equally distributed in the whole enclosure. Leftover food was removed using a tube after 5 – 10 min.

Photic conditions

For the two different experimental conditions, downwelling irradiance between 280 and 700 nm was measured in one enclosure which was specifically used for collecting irradiance data and placed at randomly chosen locations within all four outdoor tanks. Measurements were performed on 16 days under various weather conditions between 11:00 a.m. and 03:00 p.m. in February and March 2013 by using a spectrophotometer (AvaSpec 2048, Avantes) equipped with a cosine corrector (CC-UV/VIS, Avantes, Netherlands). Irradiance was calibrated against an Avantes NIST traceable application standard. For measurements, the irradiance probe was kept in a fixed position at 18 cm water depth. Fifteen single spectra were recorded within one measuring series using the software AvaSoft (Version 7.5, Avantes) and transferred to Microsoft Excel (Microsoft Office 2007) to calculate a mean spectrum. Average irradiance spectra for the two exposure treatments are shown in Fig. 1, and absolute irradiances ($W\ m^{-2}$) in the UVB (280–320 nm), UVA (320–400 nm) and PAR (400–700 nm) wavelength range are given in Table 1. The maximum amount of UVB was 0.76 $W\ m^{-2}$ (± SE 0.30 $W\ m^{-2}$) for UVB-enhanced and 0.48 $W\ m^{-2}$ (± SE 0.29 $W\ m^{-2}$) for UVB-normal which is below values measured in August 2012 during sunny conditions in the same experimental set-up (0.79 $W\ m^{-2}$). Mean values of absolute irradiance for the experimental phase were 0.33 $W\ m^{-2}$ (± SE 0.12 $W\ m^{-2}$) for UVB-enhanced and 0.21 $W\ m^{-2}$ (± SE 0.08 $W\ m^{-2}$) for UVB-normal conditions. UVB-enhanced fish were exposed to a daily dose of 6.48 $kJ\ m^{-2}$ (± SE 0.73 $W\ m^{-2}$) and an absolute dose of 440.39 $kJ\ m^{-2}$ (± SE 49.32 $W\ m^{-2}$), whereas UVB-normal fish received 3.89 $kJ\ m^{-2}$ (± SE 0.47 $W\ m^{-2}$) as daily and 264.80 $kJ\ m^{-2}$ (± SE 32.19 $W\ m^{-2}$) as the absolute dose (Table 1).
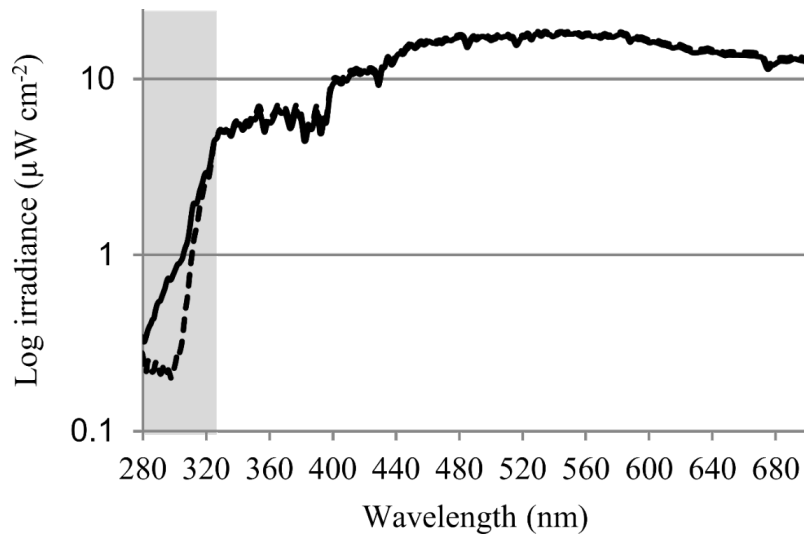
Fig. 1: Average spectral irradiance of downwelling light in a holding tank measured on 16 days under various weather conditions between 11:00 a.m. and 03:00 p.m. in February and March 2013. Displayed are the UVB range (*shaded area*), the two exposure treatments UVB-enhanced (*black solid line*) and UVB-normal (*black dashed line*).

Table 1: Maximum and mean irradiance (W m^{-2}), daily dose (kJ m^{-2}) and absolute dose (kJ m^{-2}) of ultraviolet-A (UVA) and ultraviolet-B (UVB) radiation used in the exposure treatments.

Measurement	Treatment			
	UV-enhanced		UV-normal	
Spectral range	UVB	UVA	UVB	UVA
Maximum irradiance [W m^{-2}]	0.76 (±0.30)	8.27 (±1.14)	0.48 (±0.29)	8.67 (±1.32)
Mean irradiance [W m^{-2}]	0.33 (±0.12)	3.47 (±0.61)	0.21 (±0.08)	3.69 (±0.70)
Daily dose [kJ m^{-2}]	6.48 (±0.73)	64.12 (±7.08)	3.89 (±0.47)	67.96 (±8.12)
Absolute dose [kJ m^{-2}]	440.39 (±49.32)	4360.39 (±481.51)	264.80 (±32.19)	4621.32 (±551.99)

Mean values ± SE are given

Sampling

Immune tests were conducted 66 days (04 April 2013) and 70 days (08 April 2013), respectively, after UVB lamps were switched on for the first time. One day before the tests, fish were transferred to the laboratory in their holding groups and kept in plastic tanks similar in size to the outdoor enclosures (L 39 cm × W 22 cm × H 25 cm) at a light-dark cycle of 11L:13D and a temperature of 15 ± 1 °C. At the time when fish were transferred from the outdoor tanks to the laboratory, the difference in water temperature between outdoor and laboratory conditions was between 1 and 3 °C. Illumination was provided by fluorescent tubes (Natural Daylight 5500, 36 W, 120 cm; TrueLight). Prior to dissection, the standard length (SL) (to the nearest mm) and body mass (to the nearest mg) of each individual were measured to calculate

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the body condition index (BCI) after Bolger & Connolly (1989). Changes in SL and BCI from the beginning to the end of the experimental phase were calculated for each individual separately ($\Delta SL = SL_{end} - SL_{start} / SL_{start}$; $\Delta BCI = BCI_{end} - BCI_{start} / BCI_{start}$). Fish were then killed by incision of the brain after being anaesthetized by a blow on the head. Because sexes in sticklebacks are monomorphic outside the breeding season sexes were identified histologically using gonad tissue. Head kidneys were removed and transferred into 1.5-ml tubes containing 800 µl R-90 (RPMI 1640 with 10% Millipore H_2O). Samples were stored on ice for 6 ± 1 h before being used for further analyses. The mass of the spleen, a lymphoid organ involved in adaptive immunity (Zapata *et al.*, 2006), was measured (to the nearest 0.1 mg) to calculate the splenosomatic index [SSI = 100 x spleen mass/fish mass, (Kurtz *et al.*, 2007)]. In total, 47 fish were randomly chosen out of all outdoor tanks and used for G/L ratio analyses, 24 (8 males and 16 females) of the UVB-enhanced and 23 (8 males and 15 females) of the UVB-normal treatment. From each enclosure, two sticklebacks were used, except for one enclosure of the UVB-enhanced treatment, where only one fish could be analyzed due to damage of the head kidney during dissection. Forty-six fish (8 males and 38 females) from the UVB-enhanced treatment and 56 fish (9 males and 47 females) from the UVB-normal treatment were used for calculating BCI and SSI, respectively. Due to harsh winter conditions during the experimental phase, 42 fish died, 26 from the UVB-enhanced and 16 from UVB-normal treatment. Mortality was not significantly different between treatments (Chi-square test, $N_{deathsUVB-enhanced} = 26$, $N_{deathsUVB-normal} = 16$, $\chi^2 = 2.381$, df = 1, p = 0.123). During dissection, all fish were visually checked for internal macroparasites, including an examination of the lens to check for eye flukes.

Flow cytometric analyses and granulocyte-to-lymphocyte ratio

For flow cytometric analyses, samples were transferred to the Institute for Evolution and Biodiversity at the University of Münster, Germany. Cell suspensions from head kidney leucocytes (HKL) were made forcing tissues through 40 µm nylon screen cell strainers (BD-Falcon, USA) and transferred into 96-deep-well plates. Samples were washed twice at 4 °C with R-90 and resuspended to a volume of 1 ml (Scharsack *et al.*, 2004). Differential cell numbers of HKL were determined by a flow cytometer (BD FACS Canto II; Becton Dickinson, USA) according to their forward and side scatter values (FSC/SSC characteristics). Analyses were performed using the Software FACS DIVA version 6.1.2 software (Becton Dickinson, USA). Cellular debris and aggregated cells were identified by their scatter characteristics and excluded from further evaluation. Ten microlitre of each cell suspension were supplemented with 45 µl Sheath Fluid (BD Flow), 20 µl propidium iodide solution (10 mg l^{-1}, Sigma Aldrich)

and 25 µl Latex-Beads (Beads, 30,000 / 25 µl Flow). Dead cells (propidium iodide positive) were not included in further analyses. To obtain information regarding the relative activity of the innate versus the adaptive immune system, the granulocyte-to-lymphocyte ratio (G/L ratio) was estimated by using forward and sidescatter values (FSC/SSC characteristics) to identify proportions of granulocytes and lymphocytes (for detailed methods see Scharsack *et al.*, 2004).

Statistical analyses

Analyses were conducted in R 3.3.0 statistical package (R Core Team, 2016). Data were tested for normality using Shapiro–Wilk tests. G/L ratio and SSI data were Box–Cox-transformed (Box and Cox, 1964) to meet the assumptions of normality. Linear mixed-effect models were fitted using the 'lme' function in the 'nlme' library (Pinheiro *et al.*, 2009). G/L ratio, ΔBCI and SSI were used as dependent variables, respectively. Treatment (UVB-enhanced, UVB-normal) and sex were included as fixed factors in each model. Additionally, BCI_{end} was included in the models regarding G/L ratio and SSI. The initial models included interactions between treatment and sex as well as treatment and BCI_{end}. Hierarchical random effects were used by nesting enclosure within outdoor tank as random factors. Non-significant interactions and explanatory variables were removed by using a backward stepwise model reduction (e.g. Engqvist, 2005). Therefore, the statistical significance of the interaction terms was determined by comparing the full model, including the interaction terms, to a model without the corresponding interaction term. The statistical significance of each explanatory variable was tested by removing variables in the order of their statistical relevance and comparing models with and without the variable of interest (e.g. Engqvist, 2005; Mehlis and Bakker, 2014; Hiermes *et al.*, 2015b). Tests of significance were based on likelihood-ratio tests. To test for ΔBCI deviations from zero, indicating no change, intercept models were conducted for each group separately with enclosure nested in outdoor tank as random factors. Initial standard length (SL) and ΔSL were not normally distributed and failed to respond to transformation; therefore, nonparametric Wilcoxon rank-sum tests were used to compare fish from both treatments.

Results

Body variables

Standard length (SL), body mass and body condition (BCI) did not significantly differ between the two exposure groups at the beginning of the experimental phase (SL: Wilcoxon signed-rank tests, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $W = 1606.5$, $p = 0.884$; body mass: LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $\chi^2 = 2.289$, $p = 0.130$; BCI: LME, $N_{UVB-enhanced} = 46$,

$N_{UVB-normal} = 55$, $\chi^2 = 1.179$, p = 0.278). The change in standard length (ΔSL) was significantly lower for fish from the UVB-enhanced group compared to fish from the UVB-normal group (Wilcoxon signed-rank test, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, W = 725.5, p < 0.001). Fish exposed to additional UVB showed a significantly smaller change in body condition index (lower ΔBCI) compared to fish without additional UVB (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $\chi^2 = 10.237$, p = 0.001; Table 2, Fig. 2a). Within the UVB-enhanced treatment group body condition decreased significantly (LME, $N_{UVB-enhanced} = 46$, intercept estimate = -0.029, df = 31, t = -2.609, p = 0.014; Fig. 2a), whereas no significant change in ΔBCI was observed within the UVB-normal treatment group (LME, $N_{UVB-normal} = 55$, intercept estimate = 0.016, df = 43, t = 1.559, p = 0.126; Fig. 2a). The interaction between treatment and sex as well as sex as explanatory variable did not have a significant influence on ΔBCI, and therefore, both were not included in the best-explaining model (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, all p > 0.682, Table 2).

Immune variables

Splenosomatic index was influenced by sex with males having a significantly smaller relative spleen size (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $\chi^2 = 8.043$, p = 0.005, Table 2). Independent of sex, UVB-normal fish had a significantly higher SSI compared to fish from the UVB-enhanced treatment (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $\chi^2 = 6.672$, p = 0.010, Table 2, Fig. 2b), but the interaction between sex and treatment was not significant and therefore not included in the best-explaining model (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, $\chi^2 = 0.334$, p = 0.563, Table 2) .

Furthermore, there was no significant interaction between BCI_{end} and treatment as well as no significant influence of BCI_{end} on SSI (LME, $N_{UVB-enhanced} = 46$, $N_{UVB-normal} = 55$, all p > 0.659, Table 2).

There was no significant interaction between treatment and sex as well as treatment and BCI_{end} regarding the G/L ratio (treatment x sex: LME, $N_{UVB-enhanced} = 24$, $N_{UVB-normal} = 23$, $\chi^2 = 0.068$, p = 0.794; treatment x BCI_{end}: LME, $N_{UVB-enhanced} = 24$, $N_{UVB-normal} = 23$, $\chi^2 = 1.229$, p = 0.268; Table 2). Sex and BCI_{end} were not included in the best-explaining model as they did not have a significant influence on G/L ratio (LME, $N_{UVB-enhanced} = 24$, $N_{UVB-normal} = 23$, all p > 0.057).

Sticklebacks from the UVB-enhanced treatment had a significantly higher G/L ratio (LME, $N_{UVB-enhanced} = 24$, $N_{UVB-normal} = 23$, $\chi^2 = 27.880$, p < 0.001; Table 2, Fig. 2c), representing a relatively reduced activation of the adaptive immune system.

Parasite load

All sticklebacks were free from external and internal macroparasites, and no eye flukes were observed in the lenses.
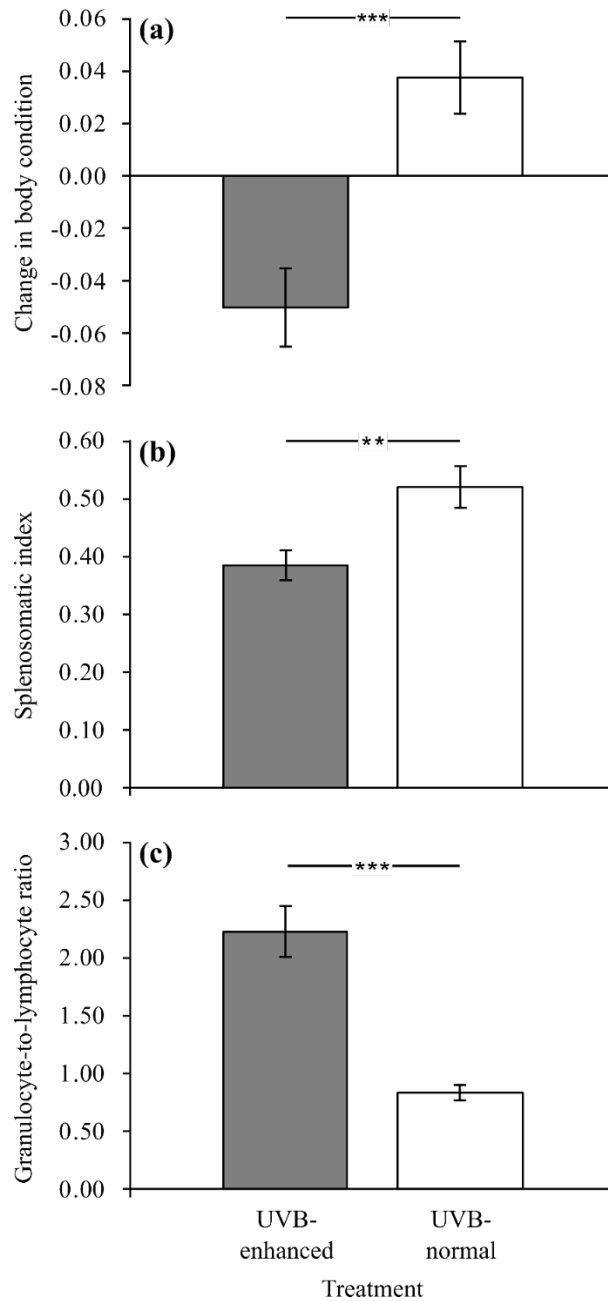

Fig. 2: Effects of the exposure treatments UVB-enhanced (*grey*) and UVB-normal (*white*) on **(a)** changes in body condition (ΔBCI), **(b)** splenosomatic index (SSI) and **(c)** the granulocyte-to-lymphocyte ratio (G/L ratio). Non-transformed data are presented for visual purposes only. Mean values ± SE are shown. ** $p < 0.01$; *** $p < 0.001$.

Table 2: All linear mixed effects models calculated. Change in body condition (ΔBCI), splenosomatic index (SSI), and the granulocyte-to-lymphocyte ratio (G/L ratio) were used as dependent variable. Treatment, sex, body condition (BCI) and the interactions between treatment and sex (Treatment x Sex) as well as treatment and body condition (Treatment x BCI_{end}) were used as explanatory variables. Hierarchical random effects were used in each model by nesting enclosure within outdoor tank as random factors. Significant results are printed in bold (p < 0.05).

Dependent variable	Explanatory variable														
	Treatment			Sex			BCI_{end}			Treatment x Sex			Treatment x BCI_{end}		
	χ^2	Δd.f.	p	χ^2	Δd.f.	p	χ^2	Δd.f.	p	χ^2	Δd.f.	p	χ^2	Δd.f.	p
ΔBCI	10.237	1	**0.001**	0.168	1	0.682	-	-	-	2.494	1	0.114	-	-	-
SSI	6.672	1	**0.010**	8.043	1	**0.005**	0.163	1	0.687	0.334	1	0.563	0.195	1	0.659
G/L ratio	27.880	1	**<0.001**	0.119	1	0.730	3.614	1	0.057	0.068	1	0.794	1.229	1	0.268

Discussion

Persistent exposure of sticklebacks to increased, but ecologically relevant, levels of UVB radiation had strong effects on body condition variables and immune function. In contrast to previous studies on long-term UVB exposure, seminatural holding and exposure conditions were chosen. Experimental animals received chronic UVB doses in form of daily exposure while remaining in their holding tanks. First, after being regularly exposed to enhanced UVB levels for two months, subadult sticklebacks showed reduced growth and loss in body condition compared to individuals that were exposed to natural solar conditions. Fish from both treatment groups were kept in equal-sized groups under identical temperature and food regimes. Consequently, the reduced growth as well as the decrease in body condition in UVB-exposed fish suggests that these individuals had to invest a higher amount of available resources in processes other than growth and somatic maintenance. Trade-offs regarding the investment in different life-history components are presumed to be the result of differential allocation of limited resources between competing physiological needs (Monaghan *et al.*, 2009) and become more pronounced under stressful conditions (e.g. Alonso-Alvarez *et al.*, 2006). Direct effects of increased UVB exposure include damage to lipids, nucleic acids and proteins (Bancroft *et al.*, 2007), whereas indirect effects are related to the formation of reactive oxygen species (ROS) (e.g. Seebacher *et al.*, 2016), leading to oxidative damage to proteins and membranes (Lesser *et al.*, 2001). Thus, exposure to UVB radiation can cause cell damage, resulting in energetically costly cell repair mechanisms or apoptosis (Groff *et al.*, 2010), which might explain the lower body condition of sticklebacks exposed to enhanced UVB levels.

In addition to the negative effects on body variables, sticklebacks from the UVB-enhanced group had a lower splenosomatic index compared to control fish. The spleen, as a lymphoid organ, plays a major role in adaptive immunity (Zapata *et al.*, 2006; Kurtz *et al.*,

2007), and spleen size has frequently been used as an intraspecific measurement of immunocompetence in studies using fish (Skarstein *et al.*, 2001; Kortet *et al.*, 2003; Ottová *et al.*, 2005). The SSI is reported to be positively related to the state of immune activation when being infected with a chronic parasite (Seppänen *et al.*, 2009), thereby referring to an increased adaptive immunity. In the present study, the SSI was found to be lower in UVB-exposed sticklebacks and consequently may indicate a reduced investment in adaptive immunity, but this requires further investigation.

Sex differences in spleen sizes, with males having smaller spleens, have been shown before in various species of birds (e.g. Møller *et al.*, 1998; Roberts *et al.*, 2004) and also in mammals (Fernández-Llario *et al.*, 2004). In birds, males are predicted to be subject to androgen-induced immunosuppression, resulting in a reduced spleen size (Møller *et al.*, 1998). However, these differences were found to be age-dependent, occurring only in sexually mature birds (Møller *et al.*, 1998). In the present study, subadult sticklebacks in a monomorphic non-reproductive stage were used. Nevertheless, given that data collection took place in early April shortly before the start of the breeding season, differences in androgen levels cannot be completely ruled out. Thus, a potentially enhanced level of testosterone in males may be responsible for the sex difference in SSI. Testosterone levels are associated with aggressiveness in fish (Li *et al.*, 2014), and the so-called pre-breeding aggression described for sticklebacks (Bakker, 1994) may be beneficial in the impending phase of territory occupation. However, the G/L ratio was not significantly influenced by sex which stays in contrast to previous studies showing that testosterone suppresses innate immunity (e.g. Kurtz *et al.*, 2007). This may suggest that males in the present study were not reproductively active.

Permanent stimulation of the innate immune system in fish from the UVB-enhanced group could have led to a shift towards innate immune responses resulting in a reduced splenosomatic index. The assumed trade-off between innate and adaptive immunity (Norris and Evans, 2000) is also supported by the higher G/L ratio in sticklebacks from the UVB-enhanced group compared to fish from the UVB-normal group. The higher granulocyte-to-lymphocyte ratio in UVB-enhanced fish indicates a relatively increased activity of the innate compared to the adaptive immune system in response to a long-term exposure to enhanced UVB radiation. Although no visible signs of skin alterations such as sunburn were observed in the present study, UVB could have induced inflammatory responses (i.e. erythema). Similar effects have been found in mammals, in which the granulocyte–macrophage colony-stimulating factor (GM-CSF) was shown to be introduced in keratinocytes after exposition to ultraviolet radiation (Imokawa *et al.*, 2015).

In fish, it has been demonstrated that inflammatory processes like those caused by enteric helminths in the digestive tract mainly involve the innate immunity (Dezfuli *et al.*, 2016). In the present study, long-term application of UVB radiation may have promoted inflammatory processes, which are accompanied by a permanent activation of the innate immune system and therefore may have led to its intensification. It is important to note that due to the fact that fish were reared in a closed tank system, the adaptive immune system was not particularly stimulated as there was no contact to parasites and contagious diseases, and even though sticklebacks were wild-caught, no internal macroparasites were observed during dissection. Reinforcing the relative activation of the innate immune system (higher G/L ratio) may be beneficial for an organism when dealing with an environmental stress factor, such as enhanced UVB. Altered immune activation in fish caused by long-term UVB radiation has been shown for juvenile Atlantic salmon where it affected the plasma immunoglobulin concentration, which is affiliated with the adaptive immune system (Jokinen *et al.*, 2008). Considering the well-studied connection between the innate and adaptive immunity (reviewed in Tort *et al.*, 2003; Magnadóttir, 2006), effects of the interactions between these two components of the immune system deserve attention and have consequences for the whole organism. Particularly with regard to photic conditions in the context of environmental change, the present study shows how enhanced UVB radiation under seminatural conditions affects the investment in innate and adaptive immunity.

In summary, the present findings reveal a UVB-induced shifted balance of the immune system towards the innate immunity. It can thus be assumed that UVB radiation as an environmental stressor has a strong impact on the defence mechanisms against viral infections and parasites by reducing the adaptive immune response. Further studies using multiple stressors are required to examine potential interactive effects of different abiotic and biotic factors on the immune system. For instance, it has been shown for juvenile Atlantic salmon that UVB radiation combined with increased temperature revealed additive effects on the plasma immunoglobulin concentration (Jokinen *et al.*, 2011). Additional demands on the innate immunity, as those observed in the present study, are very costly for the whole organism and could eventually lead to a reduced fitness as shown for mosquitoes (Ahmed *et al.*, 2002). Although resource availability was sufficient and additional stressors such as predation risk were excluded in the present study, individuals could not compensate for the negative effects of an enhanced UVB radiation. How the described effects will occur under natural conditions needs further investigation.

Acknowledgements

Chapter II

Enhanced ambient UVB radiation affects post-mating,

but not pre-mating sexual traits in a fish

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Chapter II

Enhanced ambient UVB radiation affects post-mating, but not pre-mating sexual traits in a fish

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Abstract

Organisms inhabiting shallow aquatic habitats currently experience increasing levels of solar ultraviolet B radiation (UVB). UVB causes damage on cellular and molecular levels and can affect associated life-history traits either through direct exposure or indirectly through oxidative stress generation. We examined UVB effects on pre- and post-mating sexual traits in three-spined stickleback fish (*Gasterosteus aculeatus*). Adult, reproductively non-active males were assigned to two exposure treatments under semi-natural conditions in an outdoor experiment; one group received natural radiation (UVB$_{normal}$) whilst the other group received additional UVB (UVB$_{enhanced}$). After two months, colour metrics were used to quantify male breeding colouration as pre-mating trait. At the post-mating stage, sperm morphology, number and movement as well as testes mass were determined. Males did not significantly differ in sexual ornamentation between treatments, but UVB$_{enhanced}$ fish had smaller testes as well as fewer and shorter sperm than UVB$_{normal}$ fish. Sperm movement was not significantly different between treatments. However, in UVB$_{enhanced}$ males, linear and progressive movement of sperm was positively correlated with sperm morphology (head-to-tail length ratio), whereas in UVB$_{normal}$ males this relationship was negative (but not significant). Additionally, there was a significant treatment by body condition interaction concerning head-to-tail length ratio, i.e. head-to-tail length ratio increased with condition in UVB$_{normal}$ males whereas there was no relationship in UVB$_{enhanced}$ fish. Our findings reveal that increased UVB levels influence post-mating fitness-relevant traits in males whilst having no significant impact on pre-mating sexual traits, suggesting selective UVB-effects at the gamete level with consequences for reproductive performance.

Introduction

The ongoing climate change is an anthropogenic phenomenon and focus of numerous studies (e.g. Andrady *et al.*, 2012; Williamson *et al.*, 2014; Bais *et al.*, 2015). Next to global warming, the increase in ultraviolet B radiation (UVB) reaching Earth is alarming and UVB levels are expected to remain high and even continue to rise for the next decades (Häder *et al.*, 1998; Weatherhead and Andersen, 2006). Although the Montreal Protocol reduces the emission of ozone-depleting substances (ODS) (Solomon, 2004), it is important to note that not all ODS are included (Ravishankara *et al.*, 2009; Laube *et al.*, 2014) and, furthermore, serious violations of the Montreal Protocol were observed (Montzka *et al.*, 2018). Despite the recovery of ozone in the upper stratosphere, ozone in the lower stratosphere between 60°S and 60°N is still declining since 1998, resulting in a downward trend in the stratospheric ozone column at these latitudes (Ball *et al.*, 2018). The stratospheric ozone reduction mainly impacts the UVB wavelengths (280–320 nm) which are mostly absorbed by ozone with the result that UVB reaching Earth's surface increases stronger by ozone depletion than UVA (320–400 nm), which penetrates the stratospheric ozone layer nearly completely (Bais *et al.*, 2015). Moreover, co-occurring factors like decreasing cloudiness, aerosols and air pollution influence the amount of UVR that reaches Earth's surface (Madronich *et al.*, 2015). Although these changes in photic conditions are often restricted to the polar regions, mid-latitudes are also affected (Kerr and McElroy, 1993; Madronich *et al.*, 1998; den Outer *et al.*, 2005).

Many organisms in terrestrial and aquatic habitats are susceptible to elevated levels of UVB (Häder *et al.*, 2007). In fish, UVB has detrimental effects on hatching rates (Fukunishi *et al.*, 2010), growth (Jokinen *et al.*, 2008; Vitt *et al.*, 2017a; Vitt *et al.*, 2017b) and mortality (Fukunishi *et al.*, 2006; Carreja *et al.*, 2016). Moreover, deleterious effects on cellular and molecular levels, e.g. on enzymes, DNA and RNA (Browman *et al.*, 2003; Dahms and Lee, 2010), the immune system (Jokinen *et al.*, 2001; Vitt *et al.*, 2017a) and tissue lesions (McFadzen *et al.*, 2000; Zagarese and Williamson, 2001; Groff *et al.*, 2010) have been shown. Whether environmental stress imposed by increased UVB exposure is also reflected in the expression and maintenance of reproductive traits is, however, generally unknown in fish and other taxa.

Male mating success in polyandrous species is influenced by traits for mate acquisition (pre-mating) and fertilisation (post-mating) (Parker, 1970; Andersson, 1994). The investment in elaborated traits that are more successful in sexual selection is costly (Kotiaho, 2001). Thus, male fitness in these species often depends on the allocation of resources to pre- and post-mating traits, which is expected to be affected by environmental variation (Evans and Garcia-Gonzalez, 2016). Accordingly, recent studies have investigated the impact of environmental

conditions, such as dietary resource availability (Rahman *et al.*, 2014; Mehlis *et al.*, 2015) or temperature (Breckels and Neff, 2013; Singh *et al.*, 2016) on the investment in pre- and post-mating sexual traits.

Sexually selected colour traits that are important in pre-mating sexual selection are often based on carotenoid pigments (Monaghan *et al.*, 2009). Carotenoids cannot be synthesized de novo, but need to be obtained from the diet (McGraw and Hill, 2006). Consequently, intensely coloured individuals should face higher costs in terms of an increased foraging effort (e.g. Endler, 1983). Besides their role as colour signals, carotenoids have the capacity to reduce oxidative stress caused by a shifted balance of oxidants (e.g. reactive oxygen species) and antioxidants (e.g. carotenoids) in favour of oxidants.

Carotenoid-based sexual colouration can be directly affected by UVA radiation through pigment degradation as shown for plumage colouration in great tits (*Parus major*) (Surmacki, 2008) and zebra finches (*Taeniopygia guttata*) (Blount and Pike, 2012). Indirect effects of UVA radiation on the expression of carotenoid-based male breeding colouration, probably as a result of UV-mediated oxidative stress, have been shown also in three-spined sticklebacks (*Gasterosteus aculeatus*) (Rick *et al.*, 2014). Knowledge on detrimental effects of UVB on the expression of sexually selected ornamental traits is lacking.

At the post-mating level, gonads and gametes are expected to be especially prone to UVB-induced damage. Gamete-specific effects of enhanced UVB have been shown for the freshwater zebra mussel (*Dreissena polymorpha*), where eggs and sperm are highly sensitive to UVB, leading to decreased fertilisation rates (Seaver *et al.*, 2009). In the sea urchin (*Anthocidaris crassispina*), UVB radiation caused a reduced number of motile sperm and a decreased sperm velocity resulting in a lower fertilisation success (Au *et al.*, 2002). In *A. crassispina*, enhanced UVB, but not UVA, affected the membrane integrity of sperm, whereas a combination of UVA and UVB caused impaired sperm mitochondrial function (Lu and Wu, 2005a). While these studies show effects of UVB on gametes and fertilisation rates in invertebrates, consequences for the expression of post-mating sexual traits in vertebrates remain mostly unattended.

In the present study, we examined the impact of ecologically relevant ambient UVB levels on pre- and post-mating male traits in the three-spined stickleback. Sticklebacks can typically be found in marine, brackish and freshwater habitats in the Northern Hemisphere (Wootton 1984). During the reproductive phase between April and August, fish from most populations inhabit the littoral zone of freshwater habitats, where males occupy a territory, build a nest and court receptive females (Wootton 1984). Stickleback males develop a characteristic

carotenoid-based red breeding colouration, which plays a major role in female mate choice (Bakker and Milinski, 1993). Additionally, UVA-reflective skin areas in males (Rick et al., 2004) act as visual signals in contexts such as female mate choice (Rick *et al.*, 2006) and male–male aggression (Rick and Bakker, 2008b). In contrast to the beneficial role of UVA signals in intraspecific communication, increased exposure to UVA and UVB radiation has negative consequences. For instance, non-reproductive sticklebacks exposed to enhanced levels of ambient UVB radiation during development showed reduced growth, lower body condition and an impaired immunity (Vitt *et al.*, 2017a). Long-term exposure to enhanced UVA levels had negative effects on the expression of fitness-relevant male traits including both pre-mating (male breeding colouration) and post-mating (sperm quality) traits (Rick et al., 2014).

Besides pre-mating sexual traits, post-mating sexual traits are particularly relevant in sexual selection of *G. aculeatus*, since stealing fertilisations (sneaking) is a common tactic of male sticklebacks which results in sperm competition (Largiadèr *et al.*, 2001). In a natural population, 21% of 14 observed nests contained progeny of sneaking males (Largiadèr *et al.*, 2001). Spermatogenesis in three-spined sticklebacks is largely restricted to the time span from late autumn to early winter and, therefore, the amount of sperm is limited during the breeding season (Borg, 1982). However, Sokolowska and Kulczykowska (2006) showed that spermatogenesis is not completely discontinued during spring and summer in two different populations. Stressful environmental conditions during spermatogenesis can influence male reproductive performance resulting in important fitness consequences especially under sperm competition. Examples are the reproductive performance of stickleback males raised on diets differing in quality and/or quantity (Mehlis et al., 2015) and sticklebacks exposed to different immediate temperature changes (Mehlis and Bakker, 2014).

In the present study, individual males were either exposed to elevated levels of UVB radiation (UVB$_{enhanced}$) or the natural solar light spectrum (UVB$_{normal}$) during their reproductive phase to examine the influence on the expression of sexually selected pre- and post-mating traits including physical condition. As three-spined sticklebacks overwinter in deeper water where UVB is scarce and migrate into shallower waters to breed (Wootton, 1984), UVB is most prominent during the breeding season when our experiments took place. Individuals from both treatment groups were compared with regard to the expression of their red breeding colouration (chroma, hue) as characteristic pre-mating trait, their investment in post-mating traits (testes size, number of sperm, sperm morphology, movement of sperm) and their physical condition (body condition index). We predicted that males from the UVB$_{enhanced}$ group, which face increased physiological costs in response to elevated UVB levels, show a reduced investment

in sexually selected traits. In comparison to UVA, UVB caused melanoma in a *Xiphophorus* hybrid fish model (Mitchell *et al.*, 2010) and higher mortality in Atlantic cod (*Gadus morhua*) eggs (Kouwenberg *et al.*, 1999). Thus, the effects of the higher energetic UVB radiation on sexually selected traits are possibly more pronounced than the effects of UVA (see Rick *et al.*, 2014).

Materials and methods

Study animals

Three-spined sticklebacks from an anadromous population were caught during spring migration in April 2013 on the island of Texel, the Netherlands. About 500 fish were transported to the Institute for Evolutionary Biology and Ecology in Bonn, Germany, where they were kept in one uncovered outside tank (750 l) exposed to natural sunlight with tap water supply (3 l min^{-1}) and fed with red mosquito larvae (*Chironomus* spp.) daily in excess.

The study conforms to the Association for the Study of Animal Behaviour Guidelines for the use of animals in research as well as to the legal requirements of Germany. Holding and rearing conditions were approved by the City of Bonn, Amt für Umwelt, Verbraucherschutz und Lokale Agenda, § 11 Abs. 1 TierSchG. No further licenses were required.

Experimental treatments

On 15-May-2013, 48 males (standard length: 5.8 ± 0.2 SE cm), showing first signs of nuptial colouration, were randomly selected and isolated separately in tanks, with bottom and sides made out of frosted, UV-opaque plastic (SAMLA, IKEA, Sweden) but without any cover on top. Each tank measured 39 cm x 28 cm x 28 cm (length x width x height) and was permeable to water by net-covered holes at the short sides. The 48 tanks were divided over 4 large circular outdoor tanks with a volume of 2900 l each. Thus, one outdoor tank included 12 tanks, which were dipped 20 cm into the water column, and was equipped with a filter (PonDuett 3000, 25 W, max. flow rate 1500 l / h, Pontec, Germany). Above every second of the 48 small tanks, a UVB lamp (G8T5E, 8 W (1.6 W UV), Sankyo Denki, Japan) was installed 10 cm above the water surface creating the UVB$_{enhanced}$ treatment, whereas the other tanks, representing the UVB$_{normal}$ treatment, were equipped with a dummy (grey PVC, 2 cm x 40 cm). Before the start of the experimental phase, between 1-April-2013 and 22-April-2013, seven irradiance measurements were taken on different days, during midday (11 a.m.–2 p.m.) either within the dipped plastic boxes for the UVB$_{enhanced}$ treatment (UVB lamp switched on) or the UVB$_{normal}$ treatment (dummy). Measurements were taken using a spectrophotometer (AvaSpec2048,

Avantes, the Netherlands) equipped with a cosine corrector (CC-UV/VIS, Avantes, the Netherlands) (for details, see Vitt *et al.*, 2017a) and served to ensure that the UVB levels of the UVB$_{enhanced}$ treatment did not exceed naturally occurring amounts (Fig. 1).
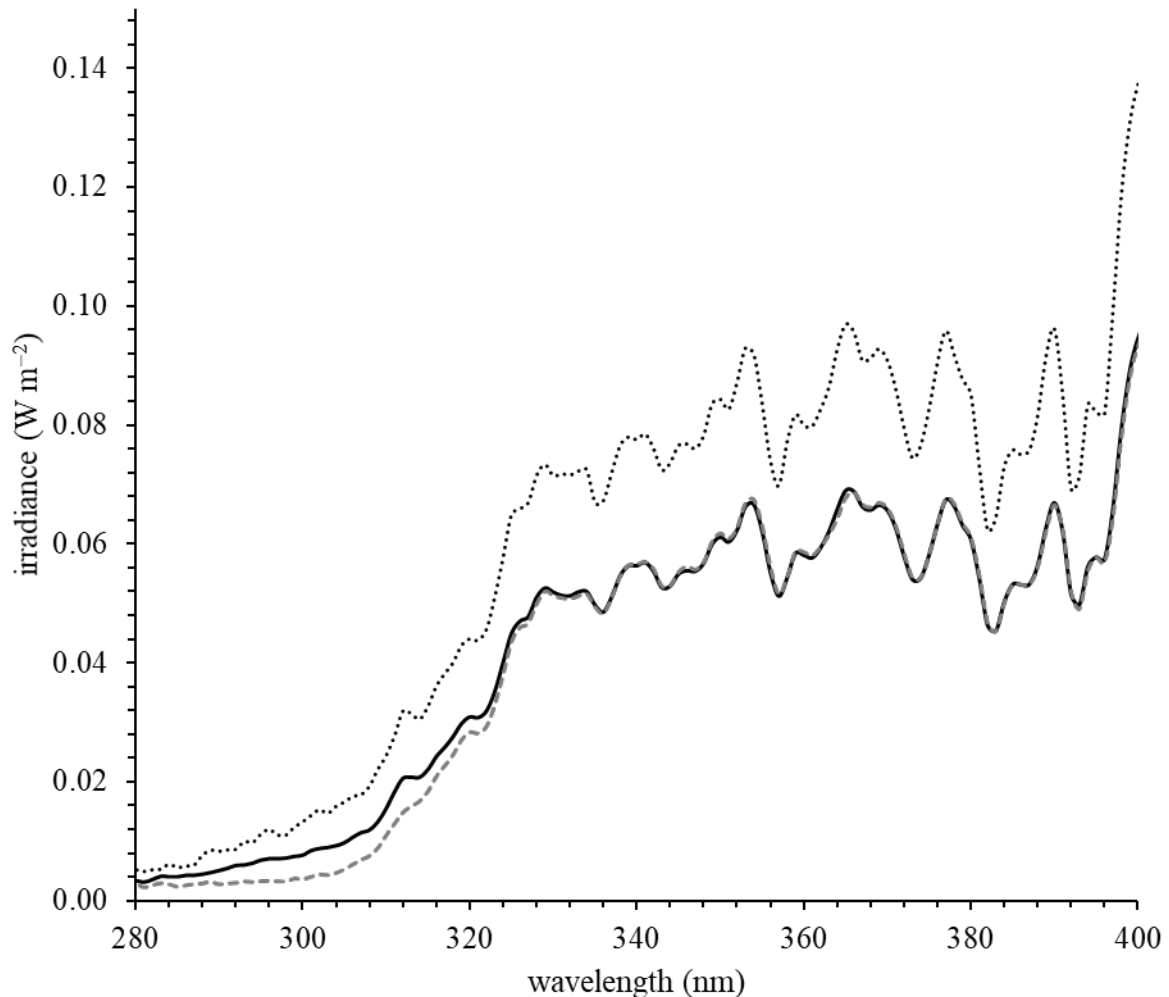

Fig. 1: Mean irradiance spectra from seven measurements per treatment (UVB$_{enhanced}$: black, solid line; UVB$_{normal}$: grey, dashed line), taken on different days in April 2013 (1-April, 2-April, 4-April, 6-April, 7-April, 11-April, 22-April). Measurements were taken in the UV wavelength range (280–400 nm) under varying cloud cover during midday (11 a.m.–2 p.m.) at a water depth of 20 cm within randomly chosen tanks of the UVB$_{enhanced}$ and UVB$_{normal}$ treatment, respectively. A representative irradiance spectrum recorded during clear-sky conditions (06-April-2013, 11 a.m.) within one tank of the UVB$_{normal}$ treatment is shown for comparison (grey, dotted line).

From 25-May-2013 until the end of the experiment on 26-July-2013, the UVB lamps were switched on during midday from 11 a.m. to 3 p.m. Throughout the whole experimental period, UVR was measured every 5 minutes using a UV data logger for long-time monitoring (UVA/UVB, UV Microlog, Scitec instruments, Bradford on Avon, GB). Data loggers were placed at the bottom of a plastic tank, equal to the holding tanks, located next to the circular outdoor tanks. The average daily doses (kJ m^{-2}) and the mean irradiance (W m^{-2}) for both, UVA

and UVB were calculated (Table 1). During the experimental period, from 15-May-2013 to 26-July-2013, experimental fish received a food ration of about 20 defrosted red *Chironomus* larvae once per day for 10 min before the remaining food was removed.

Stickleback males were given the opportunity to build a nest by providing a sand-filled petri dish (diameter 12 cm) and 3 g of green cotton threads (length 30 mm ± 10 mm; colour 597, Gütermann, Germany) on 14-June-2013. Males were stimulated daily for 15 min by a receptive female in a transparent, UV-opaque 1-l plastic box (Karlie Smart Keeper, Karlie GmbH, Germany) to build a nest (see Milinski and Bakker, 1990). Sticklebacks that failed to build a nest within 1 week were excluded from further analyses (see below). The average time span for building a nest was 3.05 ± 0.3 days (mean ± SE) for the $UVB_{enhanced}$ and 2.95 ± 0.2 days for the UVB_{normal} group.

Table 1: Mean irradiance (W m^{-2}) and daily dose (kJ m^{-2}) of ultraviolet-A (UVA) and ultraviolet-B (UVB) radiation used in the two exposure treatments.

Measurement	Treatment			
	$UV_{enhanced}$		UV_{normal}	
Spectral range	UVB	UVA	UVB	UVA
Mean irradiance (W m^{-2})	0.23	2.87	0.17	2.84
Daily dose (kJ m^{-2})	5.94	79.95	4.34	79.17

Measurement of body traits

Male standard length (SL) and body mass (BM) were determined and the residual body condition index (BCI) (Jakob *et al.*, 1996) for each male was calculated as the residual from the regression of ln BW and ln SL. Compared to the more commonly used indices (Fulton's condition factor: Le Cren 1951; relative weight: Cone 1989) the residual body condition index allows for separation of effects of body condition from effects of body size (Jakob *et al.*, 1996). Body measures were taken at the beginning, directly after isolating the males, to determine the initial body condition (BCI_{start}) and at the end of the experimental period, shortly before euthanizing them, to determine the final body condition (BCI), respectively.

Measurement of breeding colouration

From 24-July-2013 to 26-July-2013, 61 (± 1) days after isolation, each male was again stimulated with a receptive female for 15 min and then directly transferred to the laboratory using a 1-l plastic box (Karlie Smart Keeper, Karlie GmbH, Germany). Within 1 min, reflectance scans were taken using a spectrophotometer (AvaSpec 2048 fiber-optic spectrophotometer, Avantes, the Netherlands) connected to a deuterium-halogen light source (AvaLight-DHS Deuterium-Halogen Light Sources, 200–1100 nm, Avantes, the Netherlands)

for illumination (see Rick *et al.*, 2011). Reflectance was recorded relative to a 98% (300–700 nm) Spectralon white standard (WS-2, Avantes, the Netherlands) at the orange-red cheek region, directly below the left eye using a bifurcated 200 micron fiber-optic probe at a 45° angle and a fixed distance of 3 mm, ensured by a black plastic cap. Spectra were exported to Microsoft Excel file using the device-specific software with an integrated Excel output (Avasoft 7.5, Avantes, the Netherlands). The mean reflectance per male was calculated out of 20 single measurements (e.g. Rick *et al.*, 2011) and spectra were interpolated and smoothed using the program Avicol v6 (Gomez, 2006). Visual modelling was used to evaluate how male ornamentation is seen through the eyes of conspecifics. Absorbance maxima of the four cones (UV, S, M, L), provided in Rowe et al. (2004) and parameters for the calculation of visual pigment templates provided in Govardovskii *et al.* (2000) were used to calculate spectral sensitivity curves. Individual reflectance, spectral cone sensitivities and a standard daylight-simulating illumination spectrum (D65) were multiplied to obtain the absolute stimulation of each cone (see Endler and Mielke, 2005; Rick *et al.*, 2011; Hiermes *et al.*, 2016). After being converted to relative cone stimulations, values were translated to Cartesian coordinates *x*, *y* and *z* and then converted to three spherical coordinates [achieved chroma (r_A), theta and phi], representing a colour vector within a tetrahedral colour space with an achromatic centre (Endler and Mielke, 2005; Stoddard and Prum, 2008; Hiermes *et al.*, 2015a). Chroma defines the colour intensity as distance from the achromatic centre whilst greater distance means higher intensity caused by a higher concentration of carotenoids (Pike *et al.*, 2011). Comparing the maximum possible value of a hue with chroma *r* gives the achieved chroma r_A (r/r_{max}), which we used as measurement for intensity of the breeding colouration (Stoddard and Prum, 2008; Mehlis *et al.*, 2015; Hiermes *et al.*, 2016). Theta (hue-theta) is used to describe the composition of carotenoids in the red breeding colouration of the male sticklebacks as it describes the carotenoid-based colouration in the human-visible spectral range from red shifted (lower values) to orange shifted (higher values) hues (see Pike *et al.*, 2011; Hiermes *et al.*, 2016). The short-wave (UV) contribution to the perceived colour is represented by the vertical angle phi with more positive values indicating more UV perceived (see Pike *et al.*, 2011).

Measurement of testes and sperm traits

After the reflectance measurement, each male was quickly euthanised by decapitation. Subsequently, it was opened ventrally and both testes were taken out and weighed to the nearest milligram. Only the left testes were used for further analyses in the present study whilst the right testes were used in another study (Vitt et al., unpublished data). However, sperm number of left and right testes are highly correlated in sticklebacks of the study population (Bakker *et*

al., 2006) and, therefore the sperm number of one testis can be used as proxy for an individual's sperm number. For sperm trait analyses, we followed the protocol described by Mehlis *et al.* (2013); Mehlis and Bakker (2014) and Rick *et al.* (2014). The right testis was pestled in 500 µl artificial "ovarian fluid" (3.0 g NaCl, 0.1 g KCl, 0.07 g $CaCl_2$ in 1 l aqua destillata; after Elofsson et al. 2006), at a constant temperature (16 °C; Thriller, V0410E, PEQLAB, Germany). After 2 min, 3 µl of the sperm suspension was transferred to a Leja counting chamber (12 µ depth) and videotaped using a microscope (Microscope B3 Professional Series; VIDO camera CC540X; 25 fps; 320 x magnification, Motic, Germany). Each suspension was taped at ten randomly chosen positions for 3 s following a sigmoid pattern across the whole chamber (for details see Mehlis *et al.*, 2013). A sequence with the length of 1 s was chosen out of each of the ten 3-s measurements to determine sperm motility using CASA (ImageJ) resulting in the following variables: percentage of motile sperm, velocity curvilinear (VCL), velocity average path (VAP), velocity straight line (VSL), linearity (LIN), wobble (WOB), progression (PROG) and beat cross frequency (BCF). Immotile sperm were excluded using specified thresholds for VCL (10 µm s^{-1}), VAP (5 µm s^{-1}) and VSL (2 µm s^{-1}) (see also Mehlis *et al.*, 2013; Rick *et al.*, 2014). On average, 95.19 ± 9.94 (mean ± SE) single sperm per male were used for statistical analyses ($UVB_{enhanced}$: 96.29 ± 16.39; UVB_{normal} 93.86 ± 11.31). A principal component analysis (PCA) was performed (Kaiser criterion: eigenvalue > 1) to reduce sperm traits using following variables: VCL, VAP, VSL, LIN, WOB, PROG and BCF. Two components were extracted accounting for 94.98% of the variances (summarised in Table 2). Considering sample sizes, loadings higher than 0.7 were considered interpretable (Budaev, 2010). The first component (eigenvalue, 4.51; proportion of variance, 64.43%) was positively loaded with VAP, VSL, WOB, PROG and negatively with BCF. Higher values of this component, therefore, represent linear and progressive sperm movement ($PC1_{prog}$). The second component (eigenvalue, 2.14; proportion of variance, 30.55%) was positively loaded with VCL and negatively with LIN, characterising curvilinear sperm movement ($PC2_{curv}$).

Sperm suspensions for the motility measurements were also used to determine sperm number using a Neubauer improved counting chamber (0.0025 mm^2, depth 0.1 mm; Laboroptik, Germany). For each male, sperm in 64 fields was counted to calculate the total sperm number (see Mehlis *et al.*, 2012 for details). To assess a possible link between sperm morphology and movement (Humphries *et al.*, 2008), head length, mid-piece length and tail length as well as the head-to-tail length ratio [HT-ratio = (head length + midpiece-length / tail length)], were determined for 30 sperm of each stickleback using ImageJ. For this purpose, 5 µl of the suspension were fixed on a glass slide and photographed at 1000x magnification using the

program cell^D 5.1 (Olympus, Germany) and a camera (ColorView IIIu, Olympus, Germany) connected to a microscope (Olympus BX51, Germany) (for details see Mehlis and Bakker, 2013).

Statistical analyses

Five sticklebacks (UVB$_{enhanced}$ = 2, UVB$_{normal}$ = 3) failed to build a nest within one week and, therefore, were excluded from the statistical analyses. Four sticklebacks of the UVB$_{enhanced}$ and seven fish of the UVB$_{normal}$ treatment died during the experimental phase. For one male of the UVB$_{enhanced}$ group, reflectance data could not be analysed due to technical issues during the measurements. For one stickleback of the UVB$_{enhanced}$ group, no sperm could be observed during analyses and four individuals of the UVB$_{enhanced}$ group showed sperm movement below the above mentioned thresholds and were, therefore, excluded from the movement analyses. Using the Grubbs' test (Grubbs, 1969), one outlier was identified for the HT-ratio in the UVB$_{enhanced}$ group (Grubbs' test, p = 0.018) and thus morphological sperm traits of this individual were excluded from further analyses. In total, reflectance and sperm number measurements were based on 31 males (N$_{UVB-enhanced}$ = 17, N$_{UVB-normal}$ = 14), the time span for building a nest was determined for 32 (N$_{UVB-enhanced}$ = 18, N$_{UVB-normal}$ = 14), sperm movement were based on 27 males (N$_{UVB-enhanced}$ = 13, N$_{UVB-normal}$ = 14), sperm morphology on 30 males (N$_{UVB-enhanced}$ = 16, N$_{UVB-normal}$ = 14) and body and testes mass were determined for 32 sticklebacks (N$_{UVB-enhanced}$ = 18, N$_{UVB-normal}$ = 14).

Analyses were conducted in R 3.4.1 statistical package (R Core Team, 2016) and given p-values are two tailed. The time span for building a nest was not normally distributed and failed to respond to transformation; therefore, a non-parametric Wilcoxon rank-sum test was used to compare fish from both treatments. Normally distributed data were analysed using parametric tests. Linear mixed-effects models were fitted by the 'nlme' library using the 'lme' function. Treatment was used as single fixed factor in linear models with the dependent variable BCI. For the linear models with the dependent variables achieved chroma (r_A), hue-theta, phi, sperm number, sperm length, HT-ratio and percentage of motile sperm, the explanatory variables treatment and BCI were used. In addition, the interaction term of treatment and BCI was included in these models to test whether the relationship between the investment in somatic processes and post-mating traits was affected by enhanced ambient UVB. Furthermore, an interaction term consisting of PC1$_{prog}$ and treatment or PC2$_{curv}$ and treatment was used in linear models that included achieved chroma (r_A) and hue-theta as dependent variables. Treatment as fixed factor together with body mass as explanatory variable were used in a linear model with testes mass as dependent variable. The number of tracked sperm in the video analyses was used

as explanatory variable in in linear models with PC1$_\text{prog}$ and PC2$_\text{curv}$ as dependent variables. To test for differences in the relationship of sperm morphology and sperm movement between exposure treatments, the interaction between HT-ratio and treatment was used in the models regarding PC1$_\text{prog}$ and PC2$_\text{curv}$. Outdoor tank was included as random factor in each model. A backward stepwise model reduction was conducted by removing explanatory variables in the order of statistical relevance, and significance was determined by likelihood-ratio tests (see Engqvist, 2005). The residuals of the best explaining models were normally distributed, confirmed by Kolmogorov-Smirnov tests with Lilliefors correction. See Table 3 for all linear models conducted.

Table 2: Principal component analysis (PCA) of sperm traits. Listed are sperm traits, their means and standard errors and the loadings of the principal components. Components were rotated using the varimax method and scores were calculated using regression. Variable loadings of more than 0.7 are highlighted in bold and were considered as interpretable.

Sperm traits	mean	SE	PC1	PC2
velocity curvilinear (VCL)	37.789	0.814	0.359	**0.924**
velocity average path (VAP)	28.743	0.800	**0.923**	0.382
velocity straight line (VSL)	24.400	0.718	**0.976**	0.195
linearity (LIN)	0.847	0.006	0.496	**− 0.761**
wobble (WOB)	0.761	0.016	**0.870**	− 0.459
progression (PROG)	229.871	7.026	**0.948**	0.302
beat cross frequency (BCF)	11.622	0.434	**− 0.821**	0.468
sums of squares of loadings			4.509	2.138
proportion of variance explained			64.43 %	30.55 %

Results

Pre-mating traits

The physical condition of stickleback males did not differ between the two exposure groups at the start of the experiment and was not affected by exposure to enhanced UVB radiation (Table 3). Furthermore, the time span for building a nest was not significantly different between both groups (Wilcoxon signed-rank tests, N$_\text{UVB-enhanced}$ = 17, N$_\text{UVB-normal}$ = 15, W = 131, p = 0.904). Reproductively active males of the two exposure groups showed no significant difference in their red breeding colouration. Neither achieved chroma (r_A) (Table 3; Fig. 2a) nor hue-theta (Table 3; Fig. 2b) differed significantly between the two exposure groups. The colour vector phi also showed no significant difference between the individuals of the UVB$_\text{enhanced}$ and

UVB$_{normal}$ group (Table 3). All three spherical coordinates [theta, phi, achieved chroma (rA)] were not explained by BCI (Table 3). Treatment had no significant effect on the relationship between BCI and achieved chroma (r_A), hue-theta or phi (see interaction terms in Table 3).
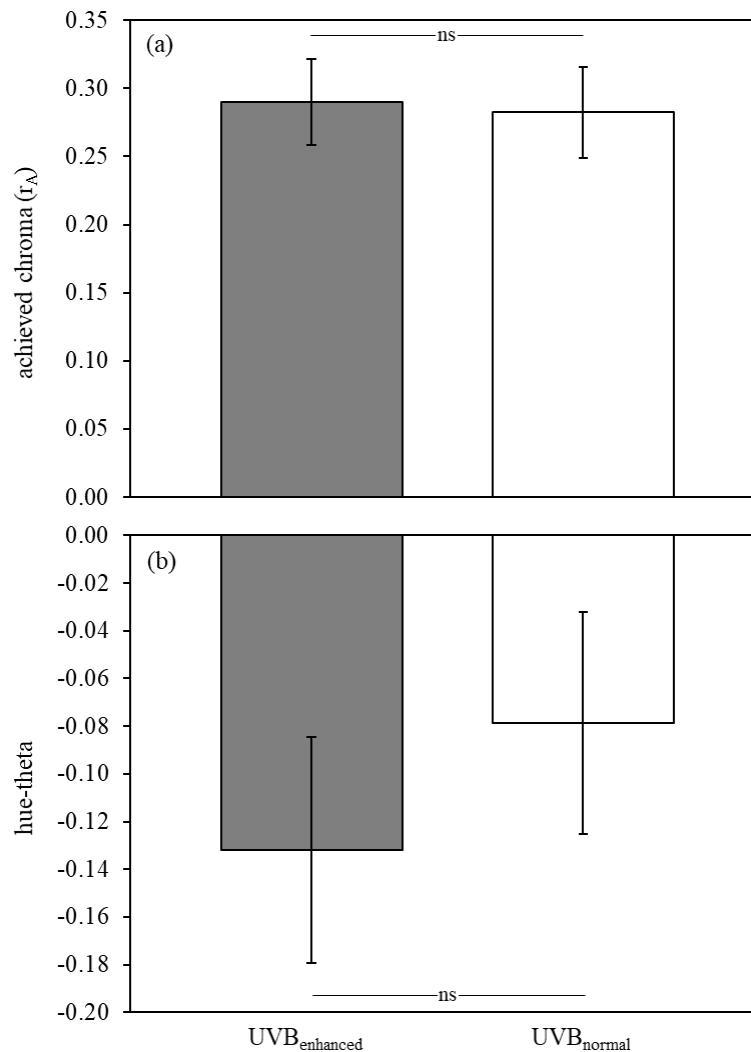

Fig. 2: Effects of the exposure treatments UVB$_{enhanced}$ (grey) and UVB$_{normal}$ (white) on the nuptial breeding colouration in form of **a)** the intensity (achieved chroma r_A) and **b)** hue of the carotenoid-based colouration (hue-theta). Mean values ± SE are shown. ns = $p > 0.05$.

Post-mating traits

Testes mass differed significantly between UVB$_{enhanced}$ and UVB$_{normal}$ groups with lighter testes in the former group (Table 3; Fig. 3a), whilst there was no significant effect of enhanced UVB on body mass (Table 3). In accordance to the heavier testes, the number of sperm was significantly higher in the UVB$_{normal}$ group (Table 3; Fig. 3b) and males of this treatment had significantly longer sperm (Table 3; Fig. 3c). The HT-ratio did not differ significantly between the two treatments (Table 3). Sperm number, sperm length and HT-ratio were not significantly related to BCI measured at the end of the experiments (Table 3).

No significant differences between treatments were found regarding the percentage of motile sperm, PC1 for linear and progressive sperm movement and PC2 for curvilinear sperm movement (Table 3). $PC1_{prog}$ and $PC2_{curv}$ were neither significantly influenced by the number of tracked sperm nor by HT-ratio (Table 3). There was no significant difference between treatments in the number of tracked sperm during movement measurements (independent two-sample t-test: $t = 0.098$, $N_{UVB-enhanced} = 13$, $N_{UVB-normal} = 14$, $p = 0.923$).

Within the $UVB_{enhanced}$ group, there was a positive relationship between progressive and linear movement ($PC1_{prog}$) and the head-to-tail length ratio (Pearson correlations: $N = 13$, $r_P = 0.801$ $p = 0.002$; Fig. 4a), whereas in the UVB_{normal} group, there was a non-significant, negative relationship (Pearson correlations: $N = 14$, $r_P = -0.335$, $p = 0.241$; Fig. 4a), resulting in a significant effect of treatment on the relationship between $PC1_{prog}$ and HT-ratio (see interaction term in Table 3; Fig. 4a). This interaction was not significant with $PC2_{curv}$ as dependent variable (Table 3).
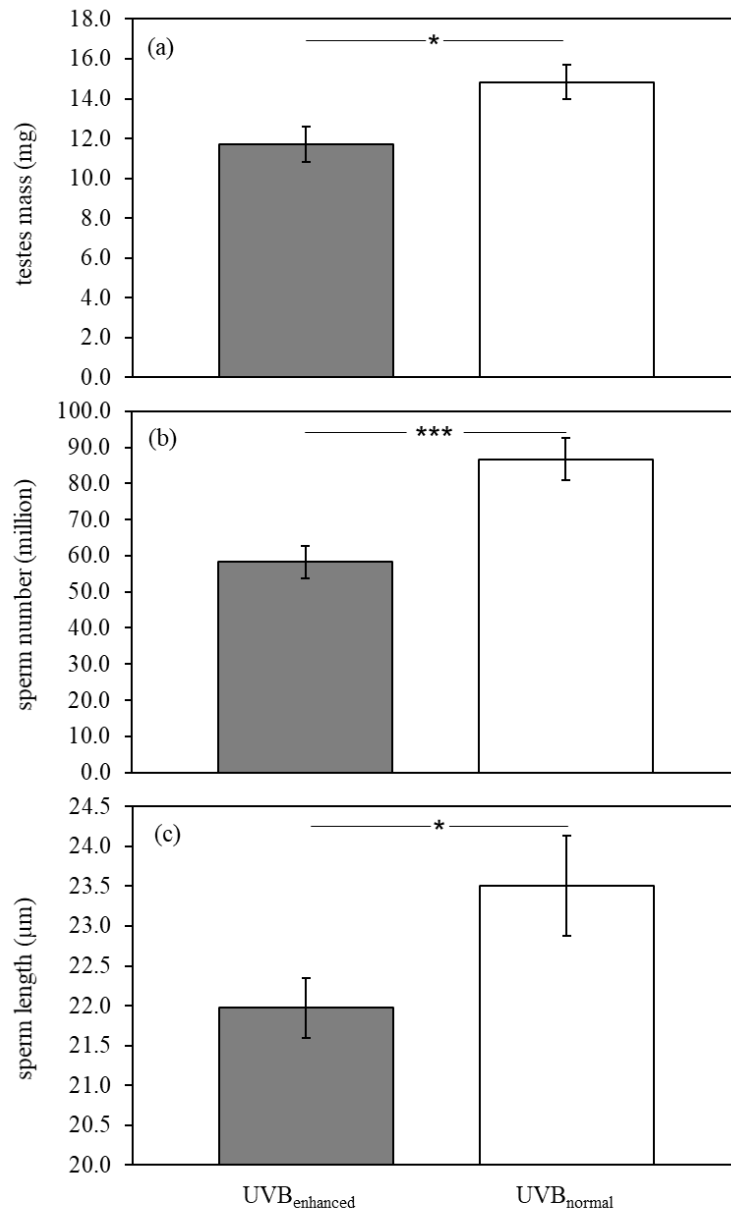
Fig. 3: Effects of the exposure treatments UVB$_{enhanced}$ (grey) and UVB$_{normal}$ (white) on sperm traits: **a)** Testes mass, **b)** number of sperm per testis and **c)** sperm length. Mean values $\pm$ SE are shown. * = p < 0.05, *** p < 0.001.

Relationship between pre- and post-mating traits

The three spherical coordinates achieved chroma (r_A), hue-theta and phi were neither significantly correlated with PC1$_{prog}$ nor with PC2$_{curv}$ (Table 3). Treatment had no significant effect on the relationship between PC1$_{prog}$ or PC2$_{curv}$ and achieved chroma (r_A) (see interaction terms in Table 3). Furthermore, there was also no significant treatment effect on the relationship between PC1$_{prog}$ or PC2$_{curv}$ and hue-theta or phi (see interaction terms in Table 3).

Treatment had no significant effect on the relationship between BCI and any testis or single or composed sperm trait (see interaction term in Table 3). Within the UVB$_{normal}$ group, there was a positive relationship between BCI and the head-to-tail length ratio (Pearson

correlations: $N = 14$, $r_P = 0.563$ $p = 0.036$; Fig. 4b), whereas in the $UVB_{enhanced}$ group there was no significant correlation (Pearson correlations: $N = 16$, $r_P = -0.181$, $p = 0.503$; Fig. 4b) resulting in a significant interaction of treatment on the relationship between BCI and HT-ratio (LME, $N_{UVB-enhanced} = 13$, $N_{UVB-normal} = 14$, $\chi^2 = 3.986$, $p = 0.046$; Table 3; Fig. 4b).
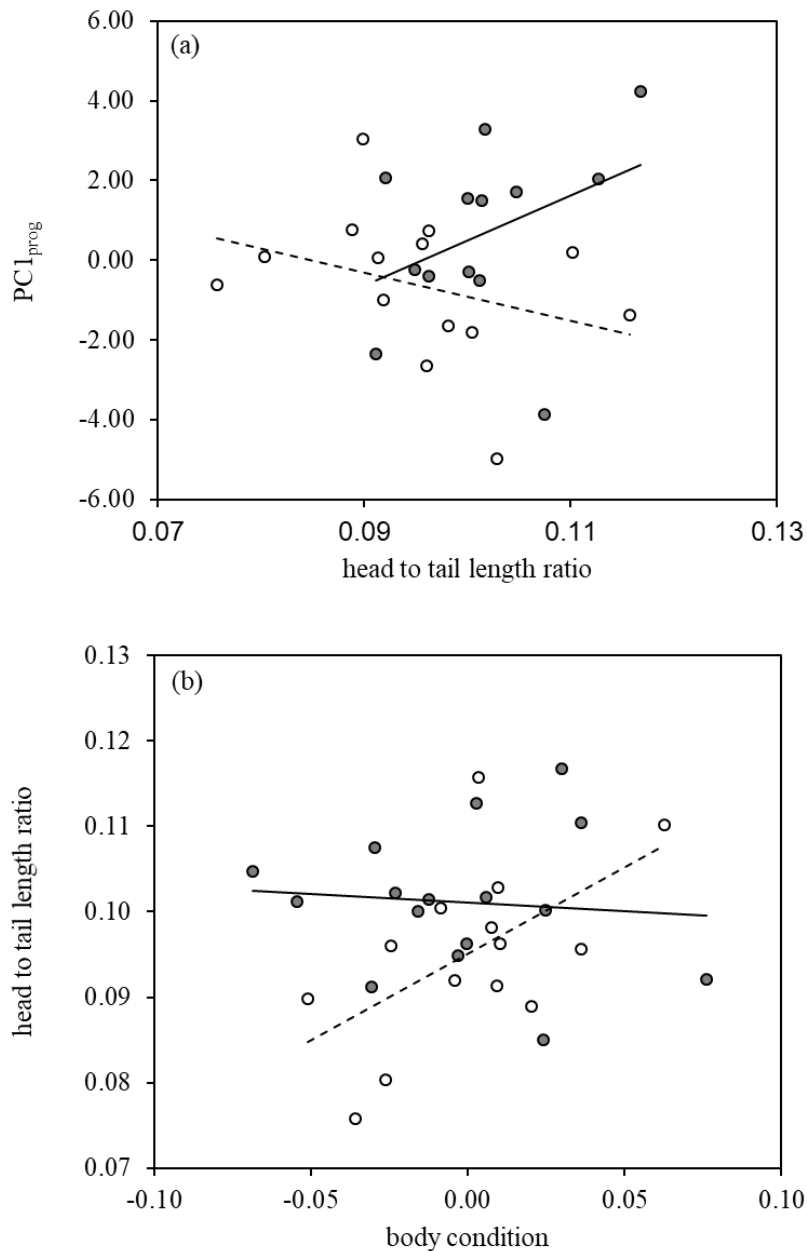

Fig. 4: a) Relationship between head-to-tail length ratio (HT-ratio) and linear and progressive sperm movement ($PC1_{prog}$) for the $UVB_{enhanced}$ group (filled circles, solid line) and the UVB_{normal} group (open circles, broken line). The relationship was significantly positive within the $UVB_{enhanced}$ group and negative, but not significantly so, within the UVB_{normal}, resulting in a significant interaction term. **b)** Relationship of body condition (BCI) and HT-ratio of males from the UVB_{normal} group (open circles, broken line) and the $UVB_{enhanced}$ group (filled circles, solid line). For the UVB_{normal} group, this relationship was significantly positive, whereas for the $UVB_{enhanced}$ group, there was no significant correlation, resulting in a significant interaction term.

Table 3: All linear mixed effects models calculated of the effects of enhanced UVB on breeding colouration and sperm and testis traits. For detailed explanation of variables, see the text. As random factor, outdoor tank was included in each linear model. x describes an interaction term. During stepwise model reduction, degrees of freedom always differed by one. Significant results are printed in bold ($p < 0.05$). BCI = body condition index, HT = head-to-tail length ratio, PC = principal component.

Dependent variable	Explanatory variable	χ^2	p
BCI_{start}	Treatment	0.369	0.544
BCI	Treatment	0.007	0.932
Body mass	Treatment	0.033	0.856
Hue-theta	BCI	0.002	0.967
	Treatment	0.578	0.447
	Treatment x BCI	< 0.001	0.987
Hue-theta	$PC1_{prog}$	1.493	0.222
	Treatment x $PC1_{prog}$	0.301	0.579
Hue-theta	$PC2_{curv}$	< 0.001	0.992
	Treatment x $PC2_{curv}$	0.376	0.540
Phi	BCI	1.707	0.191
	Treatment	3.684	0.055
	Treatment x BCI	0.115	0.734
Phi	$PC1_{prog}$	0.847	0.357
	Treatment x $PC1_{prog}$	0.297	0.586
Phi	$PC2_{curv}$	0.170	0.680
	Treatment x $PC2_{curv}$	0.332	0.564
Achieved chroma (r_A)	Treatment	0.012	0.912
	BCI	1.682	0.195
	Treatment x BCI	0.161	0.688
Achieved chroma (r_A)	Treatment	0.150	0.699
	$PC1_{prog}$	0.735	0.391
	Treatment x $PC1_{prog}$	1.405	0.236
Achieved chroma (r_A)	Treatment	0.002	0.964
	$PC2_{curv}$	3.803	0.051
	BCI	2.347	0.126
	Treatment x $PC2_{curv}$	0.258	0.612
Testes mass	Treatment x BCI	0.193	0.661
Testes mass	Body mass	2.307	0.129
	Treatment	5.832	**0.016**
Sperm number	Treatment	13.167	**< 0.001**
	BCI	2.807	0.094
	Treatment x BCI	0.457	0.499
Sperm length	Treatment	5.727	**0.017**
	BCI	0.445	0.505
	Treatment x BCI	0.254	0.614
HT-ratio	Treatment	3.311	0.069
	BCI	1.379	0.240
	Treatment x BCI	3.986	**0.046**
Motile sperm (%)	Treatment	1.636	0.200
	BCI	1.724	0.189
	Treatment x BCI	0.934	0.334
$PC1_{prog}$	BCI	0.155	0.694
	Sperm tracked	1.570	0.210
	HT-ratio	0.271	0.603
	Treatment	3.029	0.082
	Treatment x BCI	0.012	0.914
	Treatment x HT-ratio	10.736	**0.001**
$PC2_{curv}$	BCI	0.137	0.711
	Treatment	2.457	0.117
	Sperm tracked	1.235	0.266
	HT-ratio	3.151	0.076
	Treatment x BCI	0.091	0.763
	Treatment x HT-ratio	1.236	0.266

Discussion

In our study, enhanced, but ecologically relevant levels of UVB during the reproductive period had negative effects on post-mating sexual traits in stickleback males, as shown by a lower testes mass, a lower sperm number and shorter sperm. In contrast, an increased UVB exposure had no significant influence on the expression of male red breeding colouration, an important pre-mating sexual selection signal in this species. Additionally, male body condition was not significantly affected by UVB exposure. In comparison, exposure to equivalent amounts of UVB for either ca. 2 months (Vitt *et al.*, 2017a) or 7 months (Vitt *et al.*, 2017b) under a similar feeding regime during the non-reproductive stage, caused reduced growth and body condition in juvenile and subadult sticklebacks. As individuals in the present study were already adult when being assigned to the exposure groups, potential effects of UVB on body condition may be less pronounced as in subadults.

Based on a previous study showing adverse effects of enhanced UVA radiation on male breeding colouration in sticklebacks (Rick *et al.*, 2014), similar or even stronger effects would have been expected for enhanced UVB radiation in the present study. Contrary to our expectations, we found no significant differences in the expression of male breeding colouration, i.e. red intensity or hue, between individuals from the two exposure treatments. Only the short-wave (UV) contribution to the perceived colour (phi) tended to differ between males of the UVB$_{enhanced}$ and UVB$_{normal}$ group. One possible explanation for the contrasting results of the two studies could be that UVA reaches deeper layers of the skin compared to UVB (Herrling *et al.*, 2006), potentially having stronger impacts on pigment cells, mainly located in the dermis of teleost fishes (Hawkes, 1974). Furthermore, the study of Rick et al. (2014) was carried out in the laboratory, whereas the present study was conducted under outdoor conditions. Consequently, it cannot be ruled out that threat by birds reduced colour expression (Candolin, 1998), thus masking potential UVB effects, although red intensities were comparable between studies.

Exposure to enhanced UVB-levels had considerable effects on testes mass, sperm number and sperm length with UVB$_{enhanced}$ males having fewer and shorter sperm as well as smaller testes. Testes mass and sperm number are generally linked (Stockley *et al.*, 1997), also in three-spined sticklebacks (e.g. Zbinden *et al.*, 2001). Smaller testes and fewer sperm likely result in reduced effectiveness in sperm competition and reduced fertilisation success (Stockley *et al.*, 1997). In sticklebacks, sperm number is correlated with fertilisation rate in competitive and non-competitive contexts (e.g. Mehlis and Bakker, 2014). Spermatogenesis in three-spined sticklebacks is largely limited to the non-reproductive phase (Borg, 1982), but also observed

during spring and summer, although in a relatively small proportion of males (Sokolowska and Kulczykowska, 2006). Differences in sperm morphology between the two exposure treatments during the reproductive phase may, thus, have partly been due to effects of UVB on spermatogenesis. Moreover, reactive oxygen species, which represents a major cause of sperm-damage (Zan-Bar *et al.*, 2005) could be a further cause for UVB-induced differences in sperm morphology. In sea urchin sperm, UVB-induced formation of reactive oxygen species resulted in an increased lipid peroxidation (Lu and Wu, 2005b).

There was no significant association between pre-mating sexual traits (intensity and hue of male breeding colouration) and post-mating (sperm morphology and movement) traits in the present study. This result does neither support the phenotype-linked fertility hypothesis (Sheldon, 1994), which would predict a positive relationship, nor support the sperm competition theory (Parker, 1990), which would predict a negative relationship under resource limitation. The lack of a relationship in the present study was probably due to the absence of an effect of UVB on breeding colouration. In sticklebacks, Mehlis *et al.* (2015) found a positive relationship between pre- and post-mating traits under no food limitation, but a negative relationship under resource limitation (see also Pike *et al.*, 2007).

In the present study, effects of enhanced ambient UVB radiation on the relationship between sperm morphology (HT-ratio) and sperm movement ($PC1_{prog}$) were found. A positive relationship between both sperm variables in the $UVB_{enhanced}$ group was absent in individuals from the UVB_{normal} group, possibly due to UVB-associated selective constraints on sperm traits for males from the $UVB_{enhanced}$ treatment, but this requires further investigation. Responses to enhanced ambient UVB radiation at the post-mating level could be expected during active spermatogenesis, that is, mainly in autumn and winter before the reproductive season in sticklebacks. If sperm quality is phenotypically plastic, then changes may be expected as a consequence of reduced sperm number caused by enhanced levels of UVB. Phenotypic plasticity of sperm traits have been shown in Gouldian finches (*Erythrura gouldiae*) regarding sperm morphology in different social environments (Immler *et al.*, 2010) and in zebra finches, where a manipulated oxidative status resulted in a shortening of sperm mid-piece length (Tomášek *et al.*, 2017). Furthermore, in the cichlid *Telmatochromis vittatus*, sperm longevity is increased when the risk of reproductive parasitism is high (Ota *et al.*, 2010). To investigate long-term consequences, including potential counter adaptations, to increasing ambient UVB-radiation in three-spined sticklebacks, research on long-term exposure to ecologically relevant levels of UVB during growth is needed.

In summary, by exposing fish to enhanced but ecologically relevant levels of UVB under semi-natural conditions, we showed that three-spined stickleback males' sexual attractiveness, more specifically breeding colouration, was not significantly affected by enhanced UVB but we revealed adverse effects on sperm morphology. Identifying effects on functional fertility would require further studies, measuring offspring number and considering sperm competition. It is conceivable that the observed detrimental effects on post-mating traits are more pronounced under natural conditions that include direct competition for food, territories, matings and fertilisations, resulting in a substantial fitness decrease. Thus, our study highlights the impact of increasing UVB radiation during ongoing climate change on fitness-relevant traits suggesting consequences on individual's life history.

Acknowledgements

Chapter III

Differential investment in pre- and post-mating male sexual traits in response to prolonged exposure to ambient UVB radiation in a fish

Simon Vitt[*1], Theo C. M. Bakker[1] and Ingolf P. Rick[1]
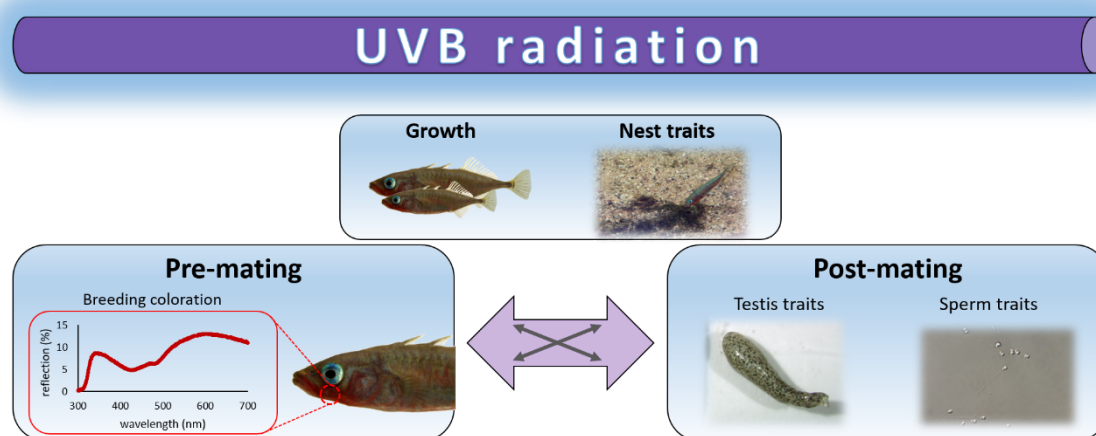
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Chapter III

Differential investment in pre- and post-mating male sexual traits in response to prolonged exposure to ambient UVB radiation in a fish

Simon Vitt, Theo C. M. Bakker and Ingolf P. Rick

Graphical abstract

Abstract

Increasing UVB radiation (UVB) reaching earth's surface following stratospheric ozone depletion is linked to serious consequences for organisms. While studies have focused on direct cytocidal and immunomodulatory effects of UVB, indirect consequences for fitness-related life-history traits are largely unexplored, although knowledge is needed to understand organismal responses to climate change. The present study investigates the effects of developmental exposure to enhanced, but ecologically relevant, ambient UVB levels on growth (body size), parental behavior (nest-building), fitness-enhancing traits of pre-mating (sexual ornamentation) and post-mating (sperm traits) sexual selection as well as their interrelationships in male three-spined sticklebacks (*Gasterosteus aculeatus*). Moreover, potentially underlying direct UVB effects, testicular antioxidant capacity (TAC) and testes and skin melanization were quantified. Juvenile full-siblings were split into two groups and raised until adulthood in a semi-natural set-up under light conditions including either natural (UVB$_{normal}$) or elevated UVB-levels (UVB$_{enhanced}$). When becoming reproductive, males were kept individually before data collection took place at their reproductive peak. The results showed that males from the UVB$_{enhanced}$-group were smaller than their brothers from the UVB$_{normal}$-treatment, whereas no treatment differences were observed for sexual ornamentation, sperm traits and TAC.

Moreover, UVB-stressed males built their nests faster and the relationship between body size and nest size was negative for UVB$_{enhanced}$-males while being positive for UVB$_{normal}$-males. These results demonstrated that, depending on physical state, UVB-stressed males adjusted their behavior to some extent. Additionally, a trade-off between the investment in pre- and post-mating traits was found for UVB$_{enhanced}$-males, i.e. the intensity of their breeding coloration was negatively correlated with sperm number whereas this relationship was reversed for UVB$_{normal}$-males, thus showing an interaction between pre- and post-mating traits regarding exposure treatment. The interaction provides first experimental evidence that differential allocation to energetically demanding pre- and post-mating components of male fitness is triggered by a key environmental stressor of climate change.

Introduction

Climate change is associated with changes in the abiotic environment, with important examples being the occurrence and increase of unseasonable, variable and extreme weather conditions (Parmesan *et al.*, 2000; Stott, 2016). Environmental stress, e.g. through increases in temperature and solar radiation, is well documented and known to have serious consequences, especially for aquatic ecosystems (Häder *et al.*, 2015; Bais *et al.*, 2019; Williamson *et al.*, 2019). Organisms can respond to environmental change, such as rising temperatures, by either shifting their habitats through dispersal (reviewed in Parmesan, 2006) or coping with the stressful conditions through genetic adaptation or phenotypic plasticity (Fuller *et al.*, 2010). Phenotypic plasticity, i.e. one genotype producing alternative phenotypes in response to the environment, represents a first non-genetic response of an individual towards environmental variation (Bradshaw and Holzapfel, 2008) and is of particular importance when studying the direct impacts of environmental change on organisms.

In addition to the profound negative consequences of climate change-induced thermal stress for aquatic organisms in general (e.g. Deutsch *et al.*, 2015), and fishes in particular (e.g. Gobler *et al.*, 2018), solar ultraviolet (UV) radiation represents a further important stressor attributed to climate change (e.g. Häder *et al.*, 2011; Williamson *et al.*, 2019). The continuing increase in ultraviolet radiation (UVR), especially in the ultraviolet B (UVB: 280–320nm) spectral region, that is reaching the Earth's surface (e.g. Laube *et al.*, 2014; Ball *et al.*, 2018), is mainly caused by anthropogenic-induced ozone depletion, but other factors such as cloud cover, aerosols and surface reflectivity also play a contributing role (Bais *et al.*, 2019). Even though the biological effects of UV radiation are less well explored compared to, for example, the effects of elevated temperatures (e.g. Carney Almroth *et al.*, 2015; Dzyuba *et al.*, 2019),

negative impacts of UVR on aquatic organisms have been shown at molecular (Dahms and Lee, 2010) and cellular (Sucré *et al.*, 2011) levels. UVB was found to increase oxidative stress levels in different species of fish (Lesser *et al.*, 2001; Carrasco-Malio *et al.*, 2014; Seebacher *et al.*, 2016; Ghanizadeh-Kazerouni *et al.*, 2017). Furthermore, UV radiation can have serious effects on gonadal tissue, thereby directly affecting fertilization success. In sea urchin (*Anthocidaris crassispina*), UVB leads to an increase in reactive oxygen species (ROS) and impaired fertilization (Lu and Wu, 2005b). In fishes, UVB-impaired motility and fertility of Nile tilapia (*Oreochromis niloticus*) sperm (Zan-Bar *et al.*, 2005). UVB caused a decreased immune function in roach (*Rutilus rutilus*) (Salo *et al.*, 2000a; Jokinen *et al.*, 2001), reduced the hatching rate of red sea bream (*Pagrus major*) and the survival of Atlantic cod larvae (*Gadus morhua*) in a predation context (Fukunishi *et al.*, 2012).

Enhanced ambient UVR is often associated with an increased formation of ROS, resulting in oxidative stress (Häder *et al.*, 2015; Birnie-Gauvin *et al.*, 2017). Oxidative stress, i.e. a shifted balance of oxidants and antioxidants towards oxidants (Birben *et al.*, 2012), negatively affects DNA, lipids and proteins (Birben *et al.*, 2012). Carotenoids, a specific class of pigments synthesized by plants, are capable to counteract oxidative stress (Stahl and Sies, 2002; 2003) and also play a role as immunostimulants (McGraw and Ardia, 2003). In many species, males show carotenoid-pigmented ornaments, whose expression functions as honest indicator of quality and determines male mating success through female mate choice in the pre-mating phase of sexual selection (Bakker, 1993; Giraudeau *et al.*, 2018; Candolin, 2019). Based on their antioxidant properties, carotenoids have a positive impact on spermatozoa (Blount *et al.*, 2001; Peters *et al.*, 2007; Pike *et al.*, 2007), which are generally prone to oxidative stress (Tomášek *et al.*, 2017), and thereby affect sperm functions that are advantageous in post-mating sexual selection in polyandrous mating systems (e.g. Pérez-Rodríguez, 2009; Rahman *et al.*, 2015). Animals cannot synthesize carotenoids *de novo* and instead must obtain them from their diet (Olson and Owens, 1998), which makes them a potential key resource for allocation trade-offs between pre-mating secondary sexual traits and post-mating ejaculate quality especially under stressful environmental conditions that influence the oxidative balance of an individual (reviewed by Blount, 2004; Mehlis *et al.*, 2015; Simmons *et al.*, 2017; Koch and Hill, 2018).

We used a teleost fish species, the three-spined stickleback (*Gasterosteus aculeatus*) to investigate phenotypic plastic responses to UVR stress. Sticklebacks inhabit shallow marine, brackish and freshwater habitats in the Northern Hemisphere (Wootton, 1984) and are naturally exposed to ambient UVR mainly consisting of ultraviolet A (UVA: 320–400nm) but also UVB radiation (Vitt *et al.*, 2019). When becoming reproductively active between April and August,

most populations inhabit the littoral zone of freshwater habitats and males express a characteristic carotenoid-based red nuptial coloration, which plays an important role in male-male competition (Bakker, 1986) and is a major criterion in female mate choice (McLennan and McPhail, 1989; Bakker and Milinski, 1993). Reproductive active males establish a territory in which they build a nest and attempt to attract females to spawn by performing a characteristic zig-zag courtship dance (Wootton, 1984). In sticklebacks, stealing of fertilizations (sneaking) occurs frequently (Largiadèr *et al.*, 2001) and results in a strong risk of sperm competition. Consequently, producing sperm of high quantity and/or quality is essential as higher sperm number as well as higher swimming abilities enhance the probability of fertilizing eggs when sperm competition is high (reviewed in Parker and Pizzari, 2010). Spermatogenesis in sticklebacks mainly takes place from late autumn to early winter (Borg, 1982) and male sperm reserves can be seen as limited during the breeding season. Thus, sperm allocation strongly impacts a male's reproductive success (e.g. Zbinden *et al.*, 2004). Consequently, male fitness in sticklebacks is determined by the expression of the carotenoid-based ornaments at the pre-mating level (Bakker, 1986; Bakker and Milinski, 1993) and the investment in sperm-related traits at the post-mating level (Cubillos and Guderley, 2000), both of which are affected by an individual's oxidative status (Pike *et al.*, 2010).

UVR is an important part of the *Umwelt* of stickleback fish. Sticklebacks can visually perceive UVA-light (Rowe et al. 2004) and use it during foraging *(*Rick *et al.*, 2012) and habitat selection under predation risk (Rick and Bakker, 2010). Sticklebacks reflect UVA (Rick *et al.*, 2004) and UVA-signals are used for intraspecific visual communication (Modarressie *et al.*, 2006; Rick and Bakker, 2008a; Hiermes *et al.*, 2015c). In addition, exposure to enhanced UVA radiation during the reproductive phase resulted in an impaired male breeding coloration as well as a decreased sperm velocity (Rick *et al.*, 2014). More recent studies demonstrated that exposure to elevated but ecologically relevant ambient UVB under semi-natural outdoor conditions resulted in decreased growth, lower body condition and an impaired immunity (Vitt *et al.*, 2017a) as well as a promoted predator-inspection behavior (Vitt *et al.*, 2017b) in subadult fish.

Here, we assigned full-sib fish to two groups and raised them under either natural sunlight or enhanced UVB in addition to the natural light spectrum. Our study aimed to investigate effects of an exposure to enhanced but ecologically relevant ambient UVB on general physical traits (standard length, body mass and dorsal brightness), nest-building traits as well as fitness-enhancing pre-mating traits (carotenoid-based breeding coloration) and post-mating traits (sperm number and quality) in male sticklebacks. Furthermore, as gonadal tissue

is especially prone to oxidative stress (Oakes and Van Der Kraak, 2003; Zhang *et al.*, 2016), the antioxidant capacity of the testes was determined. In a recent study, it was demonstrated that exposure of adult stickleback males to increased levels of UVB during the reproductive phase has negative effects, especially on the expression of post-mating, but not pre-mating sexual traits (Vitt *et al.*, 2019). While short-term environmental stress may be mitigated in some cases and sometimes the costs are expressed delayed, i.e. during later stages of life (Bizuayehu *et al.*, 2015), persistent exposure to stressful conditions often results in harmful effects on life history traits, including growth, reproduction and survival (reviewed in Eyck *et al.*, 2019). Consequently, the focus of the present study was to determine whether and how persistent, long-term UVB exposure, applied from an early juvenile stage on, affects developmental processes, especially considering sexual maturation and resource allocation to different (pre- or post-mating) traits or general body processes, e.g. melanization or body size, in reproductively active males.

Methods

Study animals / in vitro fertilization

Sticklebacks used in the present study were laboratory-bred descendants from fish kept in outside stock tank (volume 700 l; with a tap-water flow rate of $3\,l\,min^{-1}$ and air ventilation) that were caught from an anadromous population during their spring migration in March 2015 in Texel, the Netherlands.

In total 24 full-sib families were created by in vitro fertilizations in an air-conditioned room (17 ± 1°C) from 8 June to 8 July 2015. For each fertilization, testes of an adult reproductively active male were removed and pestled in one Eppendorf tube containing 500 µl of artificial "ovarian fluid" (3.0 g NaCl, 0.1g KCl, 0.07 g $CaCl_2$ in 1 l aqua dest.; after Elofsson et al. 2006). Immediately thereafter a gravid female was gently stripped and served as egg donor. Eggs were placed in a petri dish that already contained 1,000 µl of tap water. Sperm suspension was equally distributed over the eggs and after 10 min, each clutch of eggs was separately stored in a 1 l box, which was aerated by an airstone. Boxes were placed in an air-conditioned room with constant light regime and temperature (16L : 8D, 17 ± 1°C). The fry were fed daily with *Artemia* nauplii for six weeks after hatching in excess. After six weeks, individuals were transferred to holding tanks measuring 50 cm x 30 cm x 30 cm (length x width x height) at the same light regime and temperature and fed daily with red mosquito larvae (*Chironomus* spec.) in excess. Illumination was provided by natural daylight-simulating

fluorescent lamps (True-Light lamp 58W/5500 T8, True-Light International GmbH, Germany), protected with UV-opaque lamp covers.

Experimental procedure

On 2 September 2015, on average 93 days ($\pm$ 9.18 SD) after hatching, groups of 40 juvenile sticklebacks from each of 24 full-sib families were split in half and transferred to 48 enclosures measuring 39 cm x 28 cm x 28 cm (length x width x height) with a water level of 20 cm, resulting in 20 fish per enclosure. As sticklebacks form shoals during winter (Bell and Foster, 1994) we also kept them in groups to ensure more natural conditions. Twelve enclosures were mounted in one circular outdoor tank (2500 l, diameter of 2 m, AquaTech). In total, four outdoor tanks were arranged next to each other in a square of 36 m². In each outdoor tank, enclosures were arranged in a circle and a filter (PonDuett 3000, Pontec) was placed in the center. Two exposure treatments were generated by mounting a UVB lamp (G8T5E, 8 W, Sankyo Denki, Kanagawa, Japan) 10 cm above every second enclosure (UVB$_{enhanced}$) whereas the remaining enclosures were equipped with a dummy (grey PVC, 2 x 40 cm), providing similar shading as in enclosures with UVB lamps (UVB$_{normal}$). From 7 September 2015 on, the UVB lamps were switched on daily between 11 a.m. and 1 p.m. Irradiation time was reduced to 1 hour (11 a.m. to 12 midday) from 19 November 2015 to 14 April 2016 simulating UVB-winter conditions. From 14 April 2016 until the end of the experiments on 28 June 2016, exposure time was set to 2 hours again (11 a.m. to 1 p.m.). UVR was measured at an interval of 5 min using UV data loggers (UVA / UVB, UV Microlog, Scitec Instruments, Bradford on Avon, GB). Mean irradiance levels during exposure times for winter and summer conditions did not exceed natural occurring levels (Vitt *et al.*, 2017a; Vitt *et al.*, 2019) and are given in Table 1.

On 26 April 2016, one male stickleback per enclosure was randomly chosen to stay whereas the remaining fish were removed and served for another study (Vitt et al., unpublished data).

Table 1: Mean irradiance during midday and daily dose (kJ m^{-2}) of ultraviolet A (UVA) and ultraviolet B (UVB) radiation used in the exposure treatments. Winter conditions (additional UVB from 11 a.m. to 12 midday) were used from 19 November 2015 to 14 April 2016. Summer conditions (additional UVB from 11 a.m. to 1 p.m.) were created from 7 September 2015 to 19 November 2015 and 14 April to 28 June 2016, respectively.

Measurement	Winter conditions				Summer conditions			
	UVB$_{enhanced}$		UVB$_{normal}$		UVB$_{enhanced}$		UVB$_{normal}$	
Spectral range	UVA	UVB	UVA	UVB	UVA	UVB	UVA	UVB
Mean irradiance - midday (W m^{-2})	2.15	0.36	2.05	0.11	3.94	0.50	3.55	0.29
Daily dose (kJ m^{-2})	45.58	2.36	45.20	1.46	87.33	6.12	84.26	4.42

Nest-building traits

On 17 May 2016, each of the 48 males (UVB$_{normal}$: 24; UVB$_{enhanced}$: 24) received a sand-filled petri dish (diameter 12 cm) and 3 g of 30 ± 10 mm green yarn threads (color 597, Gütermann) to build a nest. In sticklebacks, there are some hints that nests serve as ornaments (Barber *et al.*, 2001; Östlund-Nilsson and Holmlund, 2003) and nest building is assumed to be costly (Wootton *et al.*, 1995). Hence, construction time for building a nest was recorded for each male (nest$_{time}$). Furthermore, nest size (nest$_{area}$) was determined using digital images and the software ImageJ 1.48. Therefore, nests were carefully taken out of the enclosures and placed next to a piece of scale paper, serving as size standard, on white styrofoam. Images were taken with a digital camera (Nikon D5000 with AF-S MicroNikkor 105-mm 1:28G macro objective) at a distance of 1 meter. A concave polygon was drawn in ImageJ enclosing only threads being intertwined within the nest or both ends were actively integrated into the nest (for details see Rushbrook *et al.*, 2008). Nests were built on average in 2.73 ± 0.32 days (mean ± SE).

Reflectance measurements and visual modelling

At the end of the experimental phase, the expression of male red breeding coloration as pre-mating trait involved in female mate choice and male-male competition was determined spectrophotometrically. First, each male was stimulated by presenting a receptive female for 15 min daily from 20 June 2016 to 28 June 2016. Immediately thereafter, reflectance measurements were taken outside the water using a spectrophotometer (AvaSpec 2048 fiber-optic spectrophotometer, Avantes, Eerbeek, the Netherlands). Light was emitted by a light source (AvaLight-DHS Deuterium-Halogen Light Sources, 200–1100 nm, Avantes), and reflectance scans of the red breeding coloration on the males' left lateral side at the cheek region (e.g. see Rick *et al.*, 2014; Mehlis *et al.*, 2015) were obtained by using a bifurcated 200-μm fiber-optic probe, held at an angle of 45 degrees towards the body surface. To ensure ambient

light was excluded and measurements were taken at a fixed distance of 3 mm and a consistent angle, a beveled black plastic cap was mounted at the probe end (e.g. see Rick *et al.*, 2014; Hiermes *et al.*, 2016). Additionally, reflectance measurements of a second body region from each male, the left dorsolateral area next to the second spine (see Rick *et al.*, 2014), were taken in order to determine the degree of melanization as a potential measure of investment in UV protection. For each region, twenty reflectance scans were taken in succession relative to a 98% (300–700 nm) Spectralon white standard (WS-2, Avantes, Eerbeek, the Netherlands) (e.g. Rick *et al.*, 2011). Spectra were exported to Microsoft Excel using the software Avasoft (Version 7.5, Avantes, Eerbeek, the Netherlands) and an integrated Excel output. For every individual, one spectrum per measured region was determined by calculating the mean over all twenty single measurements. Afterwards, the program Avicol_v6 (Gomez, 2006) was used for smoothening and interpolating the mean spectra, resulting in one value per nanometer from 300 nm to 700 nm. Brightness was calculated as the absolute total reflectance (R) between 300 and 700 nm. The UV-chroma was defined as the relative reflectance between 300 and 400 nm ($R_{300-400nm}$ / $R_{300-700nm}$). To estimate how the respective visual signals would be perceived by a conspecific, a visual modelling approach was conducted. The sticklebacks retina contains four types of cone cells, absorbing at ultraviolet (UV, 360 nm), short (S, 435 nm), medium (M, 530 nm) and long (L, 605 nm) wavelengths (Rowe *et al.*, 2004). Spectral sensitivity curves were computed using the absorbance maxima of the four cone receptors (UV, S, M, L) provided in Rowe et al. (2004) and parameters for the calculation of visual pigment templates provided in Govardovskii *et al.* (2000). By multiplying each male's reflectance spectra, a standard daylight-simulating illumination spectrum (D65) and the spectral sensitivity functions, the absolute stimulation for each cone class was calculated (see Endler *et al.*, 2005; Rick *et al.*, 2011; Hiermes *et al.*, 2016). Absolute quantum catches for the four single cone classes were converted to relative quantum catches (see Rick *et al.*, 2011) and used to obtain the Cartesian coordinates in a tetrahedral color space (x, y, z) by applying the formulae provided in Kelber *et al.* (2003). These coordinates were then converted into spherical coordinates (achieved chroma (r_A) and theta) which represent a color vector within a tetrahedral color space containing an achromatic center (Endler *et al.*, 2005; Stoddard and Prum, 2008). "Hue" describes a specific color as a direction of a color vector (Stoddard and Prum, 2008). Theta is used as a measure of the "hue" (hue-theta) by describing the human-visible wavelengths of the carotenoid-based breeding coloration of male sticklebacks with higher values for orange-shifted and lower values for more red-shifted hues (see Pike *et al.*, 2011). The distance of a point from the achromatic center within a tetrahedral color space is defined by the variable chroma representing the intensity of

the color. Achieved chroma (r_A) was used in the present study and is defined by the value for chroma in comparison to the maximum possible value for a specific hue (r/r_{max}) (Stoddard and Prum, 2008; Hiermes *et al.*, 2016).

Measurement of body variables

Directly following the reflectance measurements, standard length (SL) to the nearest millimeter and body mass to the nearest milligram were measured and used to calculate the residual body condition index (BCI) as the residuals from a regression of *ln* body mass and *ln* SL (Jakob *et al.*, 1996). The residual body condition index is described as a reliable index as it does not vary with body size (Jakob *et al.*, 1996) and is therefore more suitable than the commonly used Fulton's condition factor (Le Cren, 1951), especially for long-term studies with environmental factors potentially affecting growth. Thereafter, stickleback males were stunned by a blow on the head and quickly killed by decapitation.

Measurement of testis and sperm traits

Fish were opened ventrally, both testes obtained and weighed to the nearest milligram. Subsequently, the right testis was pestled in 500 µl artificial "ovarian fluid" (3.0 g NaCl, 0.1g KCl, 0.07 g $CaCl_2$ in 1 l aqua dest.; after Elofsson et al., 2006) at a constant temperature of 16° C (Thriller, V0410E, PEQLAB, Erlangen, Germany) and stored for 2 min. Sperm movement was videotaped using a microscope (Motic microscope B3 Professional Series; VIDO camera CC540X; 25 fps; 320 x magnification, China Group Co Ltd., Hong Kong, China) and a Leja counting chamber (12 µ depth) filled with 3 µl of the sperm suspension. Ten randomly chosen positions across the whole chamber were filmed following a sigmoid pattern (for details see Mehlis *et al.*, 2013). Out of each measurement, one randomly picked second was analyzed using the software ImageJ and the CASA plugin of Wilson-Leedy and Ingermann (2007). The following variables for sperm movement were obtained: velocity curvilinear (VCL), velocity average path (VAP), velocity straight line (VSL), linearity (LIN), wobble (WOB), progression (PROG) and beat cross frequency (BCF). Immotile sperm were classified using thresholds for VCL (10 µm s^{-1}), VAP (5 µm s^{-1}) and VSL (2 µm s^{-1}) (see also Mehlis *et al.*, 2013; Rick *et al.*, 2014). On average, 107.07 ± 7.80 (mean ± SE) single sperm per male were included in the statistical analyses (UVB$_{enhanced}$: 101.76 ± 14.15; UVB$_{normal}$:111.91 ± 7.69; mean ± SE).

Sperm number was determined following Mehlis *et al.* (2012) by using sperm suspensions from the motility measurements and counting sperm located in 64 fields of a Neubauer improved counting chamber (0.0025 mm^2, depth 0.1 mm; Labor Optik, Lancing, United Kingdom).

The melanophore pigmentation (testis$_{mel}$) was quantified as described by Mehlis *et al.* (2012). Standardized images of each testis were recorded with the program Diskus 4.6 (C.H. Hilgers Technical office, Königswinter, Germany) and a digital camera (HV-C20AMP; Hitachi Denshi Ltd., Tokyo, Japan) mounted on a stereo microscope (S8AP0, Leica, Wetzlar, Germany). Testes were placed in the same position (outside upturned) and illuminated with a cold-light lamp (KL 1500 LCD, Leica, Wetzlar, Germany). A white Munsell card (N10), placed next to the testes, served as white balance and mean L* values (CIE L*a*b* color space), ranging from black (0) to white (100), were determined for the whole area of each testis and averaged for left and right testes.

Measurement of the total antioxidant capacity

The total antioxidant capacity (TAC) of the left testis was measured using a total antioxidant capacity kit (MAK 187, Sigma-Aldrich, Taufkirchen, Germany) following the manufacturer's protocol. Standards were made by generating samples with 0 (blank), 4, 8, 12, 16 and 20 nmol/well trolox standard solution (1 nmol/µl) in a 96-well plate. Testes were homogenized in an Eppendorf tube filled with 1 ml of distilled water by using a pestle. In the next step, 100 µl of each sample and 100 µl of the Cu^{2+} working solution were poured into wells. After the solution was incubated at room temperature (20 °C) for 90 min, samples were centrifuged (Centrifuge 5417R, Eppendorf, Hamburg, Germany) at 250 x g and 4 °C for 5 min to separate solid tissue components. The supernatant was diluted 1:5 to bring values within range of kit standards. Subsequently, absorbance was measured at 570 nm (A_{570}) using a microplate reader (SpectraMax 190, Molecular Devices, San Jose, USA) and the program SoftMax Pro (version 6.5, Molecular Devices, San Jose, USA). Higher scores represent a greater total antioxidant capacity (measured in millimoles of Trolox equivalents).

Statistical analyses

Three out of 48 males died during the experimental phase (UVB$_{enhanced}$ = 2, UVB$_{normal}$ = 1). During the total antioxidant capacity analyses, four samples showed no measurable absorbance or sample readings were out of the usable range provided by the used kit (UVB$_{enhanced}$ = 1, UVB$_{normal}$ = 3), respectively. For one individual of the UVB$_{enhanced}$ treatment no sperm could be observed during analyses. In total, analyses of sperm traits were based on 44 (UVB$_{enhanced}$ = 21, UVB$_{normal}$ = 23) and on the total antioxidant capacity on 41 individuals (UVB$_{enhanced}$ = 21, UVB$_{normal}$ = 20). All other analyses were based on 45 males (UVB$_{enhanced}$ = 22, UVB$_{normal}$ = 23).

Sperm movement variables (VCL, VAP, VSL, LIN, WOB, PROG and BCF) were summarized by using a principal component analysis (PCA, Kaiser criterion: eigenvalue > 1).

The first principal component (PC1) contains 66.7% of the variance and had an eigenvalue of 4.66. It was positively loaded with VAP, VSL, LIN, WOB, PROG and negatively loaded with BCF. Accordingly, higher values of PC1 represent linear and progressive sperm ($PC1_{prog}$). The second principal component (PC2) contains 20% of the variance (eigenvalue = 1.398) and was positively loaded with VCL and, to a lesser extent with BCF, representing curvilinear sperm movement ($PC2_{curv}$). PC1 and PC2 jointly explained 86.7% of the total variance (Table 2).

Analyses were conducted in R 3.5.0 statistical package (R Core Team, 2019) and given p-values are based on two-tailed tests. Except $PC2_{curv}$, hue-theta and $nest_{time}$, all variables meet the assumption of normality, confirmed by Shapiro-Wilk tests. Hue-theta and $PC2_{curv}$ were transformed to approximate normality via a Box-Cox transformation (Box and Cox, 1964). To analyze the effects on the recorded variables, linear mixed models were conducted using the 'nlme' library and the 'lme' function. Family nested within outdoor tank were included in each linear model as random factor. We performed a backward stepwise model reduction by removing non-significant variables hierarchically on a 5% significance level (see Engqvist, 2005). Exposure treatment (treatment) was included as explanatory variable in each linear model. In linear models with the dependent variables, $nest_{time}$, $nest_{area}$, achieved chroma (r_A), hue-theta, UV-chroma, $PC1_{prog}$, $PC2_{curv}$, $testis_{mel}$, $testis_{mass}$ and TAC, the explanatory variable SL was included. Linear models regarding achieved chroma (r_A), hue-theta, $PC1_{prog}$, $PC2_{curv}$ additionally included an interaction term between SL and treatment. Sperm number, $PC1_{prog}$ and $PC2_{lin}$ together with interaction terms between sperm number, $PC1_{prog}$, $PC2_{lin}$ and treatment were included in the linear models regarding achieved chroma (r_A) and hue-theta. Furthermore, in the linear models using $nest_{time}$ and $nest_{area}$ as dependent variable, an interaction term between SL and treatment was included. Testis melanization ($testis_{mel}$) and SL as well as the interaction term of SL and treatment were included in the linear model using total antioxidant capacity (TAC) as dependent variable. The melanization of the testes ($testis_{mel}$) was statistically analyzed by including $testes_{mass}$ in addition to treatment as explanatory variable and the linear model for $testes_{mass}$ by including body weight next to treatment. Residuals of the best explaining models were normally distributed, confirmed by Shapiro-Wilk tests. All linear models conducted are summarized in Table 3 and 4.

Table 2: Principal component analysis (PCA) of sperm traits. Listed are loadings of the first principal component representing linear and progressive sperm (PC1$_{prog}$) and the second principal component representing curvilinear sperm movement (PC2$_{curv}$). Given are means and standard errors (SE). Components were rotated using the varimax method and scores were calculated using regression. Loadings of more than 0.7 or lower than -0.7 are highlighted in bold and were considered as interpretable. (N$_{UVB-enhanced}$ = 21, N$_{UVB-normal}$ = 23).

Sperm traits	Mean	SE	PC1$_{prog}$	PC2$_{curv}$
Velocity curvilinear (VCL)	38.246	0.772	0.536	**0.839**
Velocity average path (VAP)	30.988	0.859	**0.919**	0.356
Velocity straight line (VSL)	27.497	0.806	**0.947**	0.299
Linearity (LIN)	0.871	0.003	**0.740**	−0.357
Wobble (WOB)	0.809	0.013	**0.861**	−0.395
Progression (PROG)	8.071	0.032	**0.838**	−0.145
Beat cross frequency (BCF)	10.040	0.417	**−0.803**	0.418
Eigenvalue			4.666	1.398
Proportion of variance explained (%)			66.7	20
Cumulative proportion (%)			66.7	86.7

Ethics

Experiments complied with the current laws of Germany and were approved by the regional office for nature, environment and consumer protection North-Rhine Westphalia (LANUV NRW, reference no. 84-02.04.2015.A580).

Results

Body variables

Stickleback males from the UVB$_{enhanced}$ group were significantly smaller and had a lower body mass compared to individuals from the UVB$_{normal}$ group (Table 3, Fig. 1a). However, there was no significant difference in physical body condition (BCI) between fish from both treatment groups (Table 3).

Males that grew up under enhanced ambient UVB radiation reflected higher proportions of UV-light at the dorsal body surface, i.e. had a significantly higher dorsal UV-chroma compared to those of the UVB$_{normal}$ group (Table 3). In comparison, no significant differences in dorsal brightness were found between males from both exposure groups (Table 3).

Nest-building traits

Independently from body size, males of the $UVB_{enhanced}$ group finished their nests faster than males from the UVB_{normal} group (Table 3; Fig. 2a) and, independently of treatment, larger sticklebacks built nests faster (Table 3). Males from the $UVB_{enhanced}$ group had nests with a larger area ($nest_{area}$), although the difference between exposure treatments was not statistically significant (Table 3; Fig. 2b). Moreover, $nest_{area}$ was not significantly affected by SL across both treatment groups (Table 3). However, within the $UVB_{enhanced}$ group, $nest_{area}$ was negatively correlated with SL, but not significantly so (lme, N = 22, χ^2 = 1.723, p = 0.189; Fig. 3b), whereas this relationship was positive in the UVB_{normal} group, although also not significant (lme, N = 23, χ^2 = 2.735, p = 0.098; Fig. 3b), resulting in a significant interaction term across treatments (Table 3; Fig. 3b).

Pre-mating traits

Males from the two exposure treatments did not significantly differ in the expression of their orange-red breeding coloration. Neither the intensity, i.e. carotenoid concentration (achieved chroma (r_A)) (Table 4; Fig. 1b) nor the carotenoid composition, hue-theta (Table 4) showed significant differences between the $UVB_{enhanced}$ and UVB_{normal} group.

Post-mating traits

Neither progressive movement ($PC1_{prog}$; Table 4; Fig. 1d) nor curvilinear movement of sperm ($PC2_{curv}$; Table 4) were significantly different between males from the $UVB_{enhanced}$ and UVB_{normal} group. There was also no significant difference between exposure treatments regarding the number of sperm (Table 4; Fig. 1c), but sperm number was significantly positively correlated with standard length (Table 4).

Exposure to enhanced levels of UVB radiation did not significantly affect the melanization or mass of the testes (Table 4). In addition, there was no significant difference in testicular levels of total antioxidant capacity (TAC) between the two exposure groups $UVB_{enhanced}$ and UVB_{normal} (Table 4) suggesting that enhanced UVB during growth does not affect testicular oxidative status. However, across both treatment groups TAC levels were significantly negatively correlated with $testis_{mel}$ (Table 4; Fig. 4). Thus, a higher antioxidant capacity of the testes was reflected in a higher level of melanophore pigmentation, i.e. lower levels of $testis_{mel}$.

Relationship between body variables, pre- and post-mating traits

Carotenoid composition (hue-theta) of the orange-red throat coloration was positively correlated with total sperm number (Table 4) which means that males with a more orange-

shifted nuptial coloration had higher sperm numbers, independently from exposure treatment. In addition, orange-red color intensity (achieved chroma (r_A)) was correlated with sperm number, but not significantly so ($N_{UVB-enhanced} = 22$, $N_{UVB-normal} = 23$, $\chi^2 = 3.724$, p = 0.054; Table 4). Statistical analyses of the association between pre- and post-mating traits revealed significantly different relationships of the intensity of the orange-red throat (achieved chroma (r_A)) and sperm number between males from both exposure groups. Within the $UVB_{enhanced}$ group, achieved chroma (r_A) was significantly negatively correlated with sperm number (lme, N = 22, $\chi^2 = 9.858$, p = 0.002; Fig. 3a) whereas this relationship was positive in the UVB_{normal} group, but not significantly so (lme, N = 23, $\chi^2 = 1.289$, p = 0.256; Fig. 3a), resulting in a significant interaction term across treatments (lme, $N_{UVB-enhanced} = 22$, $N_{UVB-normal} = 23$, $\chi^2 = 7.064$, p = 0.008; Table 4; Fig. 3a). The relationship of carotenoid composition (hue-theta) and sperm number did not differ between males from both exposure groups. Furthermore, the relationships between achieved chroma (r_A) as well as hue-theta and $PC1_{prog}$ and $PC2_{lin}$, respectively, was not significantly affected by enhanced ambient UVB levels (Table 4). Moreover, relationships between SL and achieved chroma (r_A), hue-theta, $PC1_{prog}$ and $PC2_{lin}$ were not significantly different between exposure treatments (Table 4).
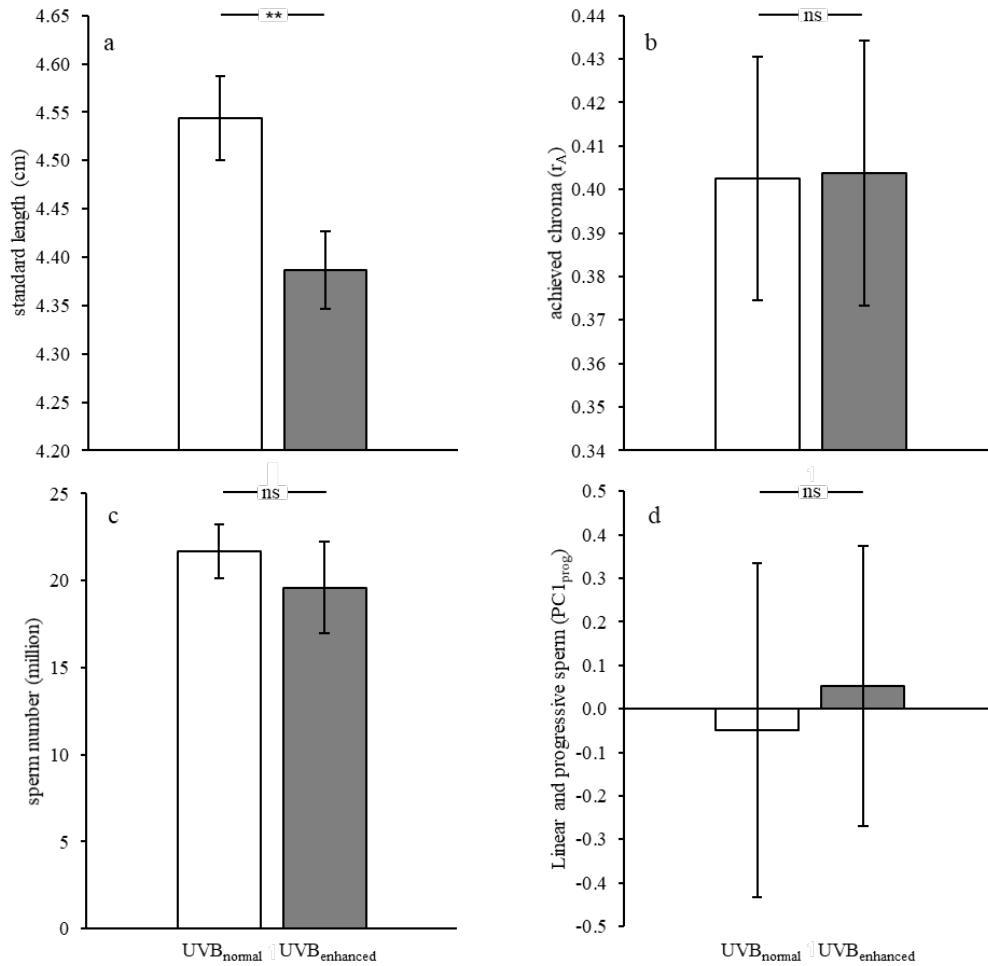
Fig. 1: Differences between the UVB_{normal} group (white bars) and the $UVB_{enhanced}$ group (grey bars) regarding **a)** the physical trait standard length, **b)** the intensity of the red breeding coloration (achieved chroma r_A) as a pre-mating trait and the post-mating traits **c)** sperm number and **d)** linear and progressive sperm movement ($PC1_{prog}$). Given are mean values and SE. ns = p > 0.05, * = p < 0.05, ** = p < 0.01.
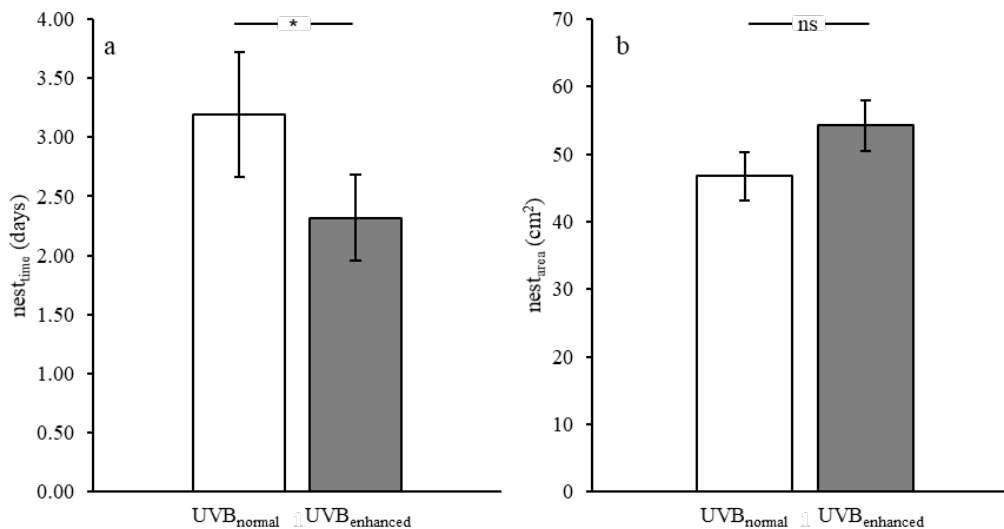

Fig. 2: a) Time for building a nest and **b)** area covered by the nest for the UVB_{normal} group (white bars) and the $UVB_{enhanced}$ group (grey bars). Given are mean values and SE. ns = p > 0.05, * = p < 0.05.
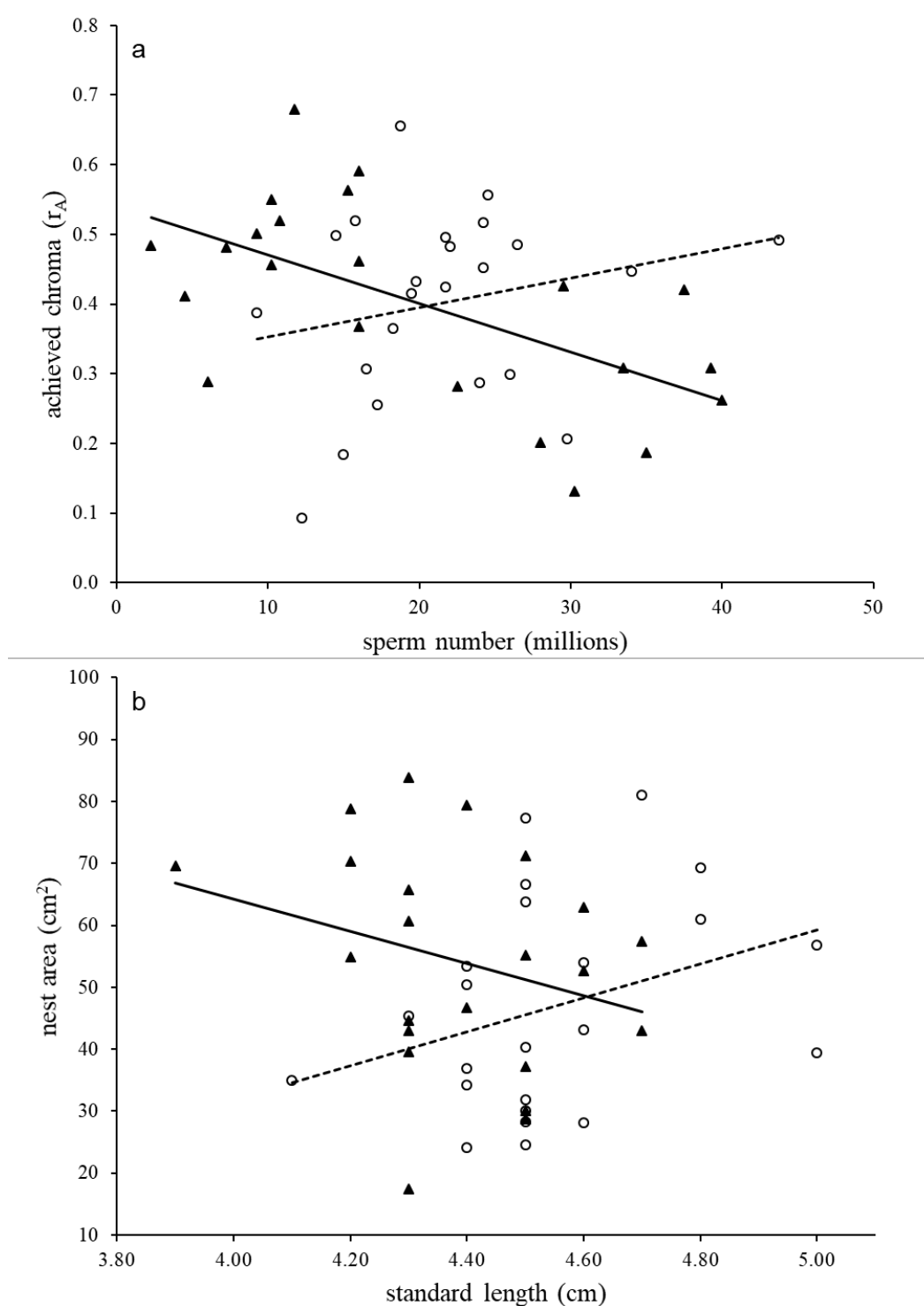
Fig. 3: a) Exposure to enhanced levels of ambient ultraviolet B radiation (UVB$_{enhanced}$, filled triangles, solid line) resulted in a significantly negative relationship between the pre-mating trait red intensity of the breeding coloration (achieved chroma (r_A)) and the sperm number. In the control group, exposed to natural sunlight only (UVB$_{normal}$, open circles, broken line), the relation between achieved chroma (r_A) and sperm number was positive, but not significant, resulting in a significant interaction term across both exposure treatments (lme, $\chi^2 = 7.064$, p = 0.008). **b)** Relationship between standard length (cm) and nest area (cm^2) for the UVB$_{enhanced}$ group (filled triangles, solid line) and the UVB$_{normal}$ group (open circles, broken line). Within the UVB$_{enhanced}$ group, the relation was negative, but not significant and within the UVB$_{normal}$ group, the relation was positive, but not significant. There was a significant interaction of treatment on the relationship between standard length and nest area (lme, $\chi^2 = 3.984$, p = 0.046).
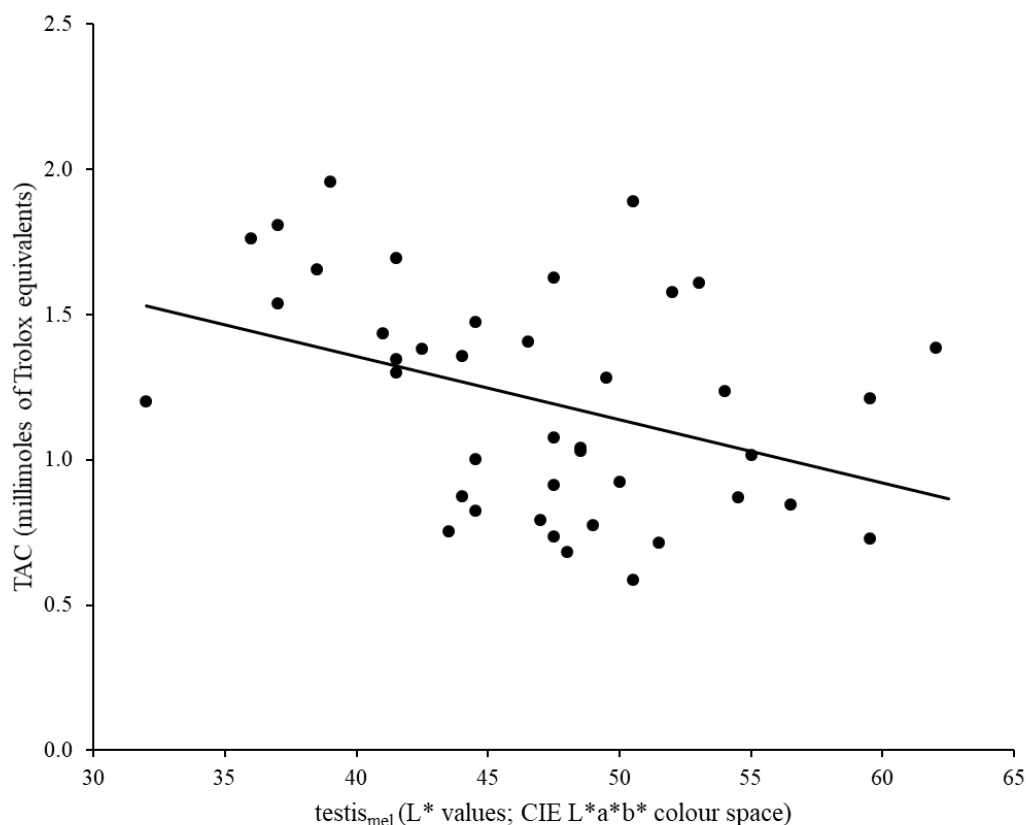
Fig. 4: Relationship between the total testicular antioxidant capacity (TAC) and the intensity of melanophore pigmentation (testis$_{mel}$), determined as mean L* value within the CIE L*a*b* color space, ranging from 0 (black) to 100 (white). Brighter testes showed lower TAC (lme, $\chi^2 = 6.602$, p = 0.010).

Table 3: Linear mixed effects models calculated for body variables and nest-building traits. As random factors, family nested within outdoor tank were included in each linear model. x describes an interaction term. A stepwise model reduction was performed and the degrees of freedom always differed by one. Significant results are printed in bold (p < 0.05).

	Dependent variable	Explanatory variable	χ^2	p
Body variables	Standard length	Treatment	8.038	**0.005**
	Body mass	Treatment	4.040	**0.044**
	Body condition index	Treatment	1.683	0.195
	Dorsal brightness	Treatment	0.078	0.780
	Relative dorsal UV reflection (UV-chroma)	Standard length	0.779	0.377
		Treatment	11.413	**0.001**
Nest-building traits	Timespan for building a nest	Standard length x treatment	0.734	0.392
		Treatment	4.803	**0.028**
		Standard length	8.764	**0.003**
	Area of the nest	Standard length x treatment	3.984	**0.046**
		Standard length	0.039	0.843
		Treatment	2.425	0.119

Table 4: Linear mixed effects models calculated for pre- and post-mating traits. As random factors, family nested within outdoor tank were included in each linear model. x describes an interaction term. A stepwise model reduction was performed and the degrees of freedom always differed by one. Significant results are printed in bold ($p < 0.05$). $PC1_{prog}$ is the first principal component representing linear and progressive sperm and $PC2_{curv}$ the second principal component representing curvilinear sperm movement, $testis_{mel}$ is the intensity of melanophore pimentation of the testes, $testes_{mass}$ is the mass of the testes, SL is standard length, TAC is testicular antioxidant capacity, and x means interaction between the concerning factors.

	Dependent variable	Explanatory variable	χ^2	**p**
Pre-mating traits	Achieved chroma (r_A)	SL x treatment	0.608	0.895
		SL	1.338	0.512
		Sperm number x treatment	7.064	**0.008**
		Treatment	0.032	0.859
		Sperm number	3.724	0.054
	Achieved chroma (r_A)	$PC1_{prog}$ x treatment	0.234	0.628
		Treatment	0.226	0.893
		$PC1_{prog}$	0.358	0.550
		SL	2.034	0.154
	Achieved chroma (r_A)	$PC2_{lin}$ x treatment	0.299	0.584
		Treatment	0.284	0.868
		$PC2_{lin}$	0.610	0.435
	Hue-theta	SL x treatment	2.056	0.195
		Sperm number x treatment	2.063	0.152
		SL	0.014	0.907
		Treatment	0.322	0.571
		Sperm number	6.176	**0.013**
	Hue-theta	$PC1_{prog}$ x treatment	0.036	0.850
		$PC1_{prog}$	0.013	0.908
		Treatment	0.115	0.734
		SL	1.591	0.207
	Hue-theta	$PC2_{lin}$ x treatment	1.051	0.305
		$PC2_{lin}$	0.213	0.644
Post-mating traits	$Testis_{mel}$	$Testes_{mass}$	<0.001	0.983
		Treatment	0.065	0.798
		SL	0.649	0.421
	$testes_{mass}$	Treatment	0.019	0.892
		Body mass	23.540	**<0.001**
	TAC	$Testis_{mel}$ x treatment	0.325	0.569
		Treatment	0.602	0.438
		SL	2.971	0.085
		$Testis_{mel}$	6.602	**0.010**
	Sperm number	Treatment	0.238	0.626
		SL	10.728	**0.001**
	$PC1_{prog}$	SL x treatment	0.158	0.692
		Sperm number	0.102	0.750
		Treatment	2.063	0.151
		SL	1.056	0.304
	$PC2_{curv}$	Sperm number	0.258	0.611
		SL x treatment	2.751	0.097
		SL	0.033	0.857
		Treatment	0.057	0.811

Discussion

Exposure to enhanced, but ecologically relevant, levels of ambient UVB radiation from early-life on resulted in a trade-off between the investment in a pre-mating (color intensity) and a post-mating (sperm number) trait. Moreover, individuals of the UVB$_{enhanced}$ group showed reduced growth and a higher dorsal UV-reflection than their full-sib brothers of the control group. Interestingly, despite being smaller and lighter, UVB$_{enhanced}$ males built their nests significantly faster than UVB$_{normal}$ males. In addition, the relationship between standard length and nest size was significantly different between males of the UVB$_{normal}$ (positive) and the UVB$_{enhanced}$ (negative) group. Direct effects of enhanced UVB on pre- and post-mating traits could not be observed.

Reproductively active males that were exposed to enhanced levels of ambient UVB radiation for 42 weeks were smaller and of lower body mass compared to males exposed to natural solar conditions. The smaller size of full-sib brother of the UVB$_{enhanced}$ group is in line with previous studies investigating the influence of enhanced ambient UVB radiation on three-spined sticklebacks from the same population (Vitt *et al.*, 2017a; Vitt *et al.*, 2017b). Accordingly, a reduced growth as a consequence of UVB has also been shown in juvenile Atlantic salmon (*Salmo salar*) (Jokinen *et al.*, 2008) and larval anchovy (*Egradulis mordax*) (Hunter *et al.*, 1981). As males of both groups were exposed to identical temperatures and received the same amount of food, differences in size and body mass might be the result of a shifted investment of available resources towards processes other than growth. In aquatic organisms, UV causes damage on lipids, nucleic acids, proteins (Bancroft *et al.*, 2007), membranes (Lesser *et al.*, 2001) and promotes the formation of reactive oxygen species (Häder *et al.*, 2015; Seebacher *et al.*, 2016). Additionally, for sticklebacks UVB radiation was shown to affect the immune system (Vitt *et al.*, 2017a). Consequently, those effects are followed by energetically costly repair mechanisms (Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010), which could explain the observed difference in body length and mass between stickleback males of the UVB$_{normal}$ and the UVB$_{enhanced}$ group. Alternatively, or in addition, negative UVB effects on digestion may explain effects on growth as it has been shown for larval European whitefish (*Coregonus lavaretus*) (Ylönen *et al.*, 2004) and the intertidal fish *Girella laevifrons* (García-Huidobro *et al.*, 2017).

Individuals exposed to enhanced ambient UVB showed a higher dorsal UV-reflection (UV-chroma) whereas there was no significant difference in total brightness between both exposure groups. Deleterious effects of UVR on internal tissue can be counteracted by absorbing, scattering or reflecting high energetic UV wavelengths in outer layers of the skin.

Melanin pigments play an important role in protective adaptations towards UVR (Debecker *et al.*, 2015) and an UV-induced increased synthesis of melanin, resulting in a darker coloration, has been shown in various taxa (Brenner and Hearing, 2008; Hu *et al.*, 2013; Debecker *et al.*, 2015; Franco-Belussi *et al.*, 2016). Surprisingly, no differences in dorsal brightness were observed between both exposure groups. Whether the enhanced dorsal UV-reflection in UVB-exposed males, which is predominantly based on dermal structural coloration (Lythgoe and Shand, 1989), provides protection of deeper skin structures as discussed by Cope et al. (2001) requires further study.

Enhanced UVB radiation had no significant effect on the expression of breeding coloration. Although a previous study showed direct negative effects of UVA on the breeding coloration of adult male sticklebacks in a laboratory setup (Rick *et al.*, 2014), such effects were absent in the present study using long-term UVB exposure in an outdoor setup. Effects on color traits caused by less energetic UVA but not by the higher energetic UVB radiation seem counterintuitive, but the longer wavelengths of the UVA radiation allow penetration deeper into the skin (Herrling *et al.*, 2006) and thereby may exert stronger damage in the dermis containing the main pigment cells (Hawkes, 1974). Accordingly, a study on UVB effects using adult males of the same study population and a comparable experimental approach as used here found no effects of enhanced ambient UVB on the expression of male breeding coloration when exposing adult fish to enhanced UVB for two months (Vitt *et al.*, 2019).

At the post-mating level, no significant differences between individuals from the UVB$_{enhanced}$ and the UVB$_{normal}$ group could be observed except that sperm number tended to be lower in males from the UVB$_{enhanced}$ group. These findings differ from the results of a similar study where strong effects on post-mating variables, e.g. reduced testes mass, lower sperm number and shorter sperm, were found when exposing reproductively active stickleback males to comparable amounts of enhanced ambient UVB for two months (Vitt *et al.*, 2019). Moreover, sperm of blue tilapia (*Oreochromis aureus*) show impaired motility and fertility (Zan-Bar *et al.*, 2005) as a consequence of direct UV exposure and UV radiation was found to have even more acute effects in Stinging Catfish (*Heteropneustes fossilis*) where direct irradiation of sperm resulted in genetic inactivation (Godwin *et al.*, 2009). In the present study, exposure of individuals to enhanced ambient UVB radiation for about nine months during growth may trigger plastic responses. Plasticity may potentially compensate for negative effects of enhanced UVB at the post-mating level, at least to a certain degree, potentially linked to negative effects on other processes like growth. However, we could not find differences in the investment in

post-mating traits at the expense of growth as UVB exposure did not cause different relationships between length and PC1$_{prog}$ or PC2$_{curv}$.

Spermatogenesis represents a fitness-relevant process during which potential compensatory mechanisms followed by environmental stress could occur. In sticklebacks, spermatogenesis is primarily restricted to the non-reproductive phase during autumn and winter (Borg, 1982). Thus, adaptions should mainly occur if the stress is present during this time span. During the breeding season, deleterious effects on already developed sperm cells may not be completely counteracted and therefore potentially reduce sperm quality. Accordingly, negative effects on sperm quality were shown for reproductive active sticklebacks being exposed to enhanced levels of UVA (Rick *et al.*, 2014) or UVB (Vitt *et al.*, 2019) with exposed individuals showing impaired sperm function. Here, potential UVB-effects on sperm, e.g. increased apoptosis, may have been compensated, e.g. by promoted spermatogenesis in the UVB$_{enhanced}$ group, which could explain the absent differences between both exposure groups.

The total antioxidant capacity (TAC) of the testes did not differ between UVB$_{normal}$ and UVB$_{enhanced}$ males. In terms of resource allocation, males may have compensated effects on TAC at the expense of other fitness-relevant traits (e.g. growth) as discussed above for absent effects on breeding coloration. In addition, enhanced levels of ambient UVB radiation did not affect testicular melanization (see also Rick et al. 2014). There was, however, a positive relationship between defence against oxidants (higher TAC) and melanization, independent from exposure to UVB. The melanization of the testis is often assumed to be associated with the protection of gonadal tissue against oxidative stress (Plonka *et al.*, 2009; Galván *et al.*, 2011).

When focusing on the relationship between the investment in pre- and post-mating traits, there was a positive, although not significant, association between the intensity of the red breeding coloration (r$_A$) and sperm number in the UVB$_{normal}$ group whereas a significant negative correlation was found in the UVB$_{enhanced}$ group, resulting in a significant interaction term across both treatments. A positive correlation between male's sexual ornamentation (achieved chroma (r$_A$)) and functional fertility (higher sperm number) is consistent with the phenotype-linked fertility hypothesis (PLFH) by Sheldon (1994) which suggests that the honesty of sexual ornaments may be maintained through a link with fertility (see Mehlis et al. 2015 for discussion and examples). On the contrary, stressed individuals seem to invest resources in a pre-mating trait (achieved chroma (r$_A$)) at the expense of a post-mating trait (sperm number) (cf. Mehlis et al. 2015), which potentially allows them to maintain their sexual attractiveness as coloration was not significantly different between both groups. This

contradicts the PLFH and, in consequence, the honesty of the red breeding coloration in males cannot be maintained in the UVB$_{enhanced}$ group. In accordance, Pike (2007) showed for sticklebacks on a low-carotenoid diet a shifted investment towards coloration at the expense of body carotenoids followed by negative fitness consequences as they completed significantly fewer breeding cycles and had a shorter lifespan. A negative relationship is predicted by sperm competition theory (SCT) (Parker, 1990) which proposes that males compensate for a limited access to matings, e.g. through less pronounced sexual ornaments, by an increased investment in spermatogenesis at the expense of pre-mating traits.

In the present study, males with a more orange-shifted coloration (higher hue-theta) had a higher sperm number, which was independent from UVB exposure. A more orange-shifted breeding coloration could be a result of reduced availability of certain high-quality carotenoids, e.g. astaxanthin (Pike *et al.*, 2011). Accordingly, stickleback males fed with normal red mosquito larvae had a significantly more orange-shifted breeding coloration in comparison with males fed astaxanthin-enriched red mosquito larvae (Mehlis *et al.*, 2015). As studies on different stickleback populations indicate that the red hue of the throat is linked to higher male quality (Bakker and Milinski, 1993; Wedekind *et al.*, 1998), orange-shifted coloration can be assumed to be less attractive. More orange colored males might thus compensate their reduced attractiveness by producing higher numbers of sperm.

Independent from treatment, larger males built their nest faster than smaller ones. Potentially, either larger males carry more nest material at once, or they build the nests more continuously, i.e. with less interruptions, resulting in less construction time. However, independent from standard length, UVB$_{enhanced}$ males built their nests significantly faster than UVB$_{normal}$ males whereas nest size was not significantly different between groups. While the relation between body size and nest area tended to be positive for UVB$_{normal}$ males, this relationship was negative, but not significantly, for UVB$_{enhanced}$ males, resulting in a significant interaction term. Preliminary evidence suggests that sticklebacks' nests might play a role in mate attraction (Barber *et al.*, 2001; Östlund-Nilsson and Holmlund, 2003). Thus, UVB$_{enhanced}$ males may benefit from less construction time by enhancing the chance of gaining matings. Furthermore, a smaller male building a nest of a similar size as a larger male may be more attractive to females as it suggests a good physical condition. A positive relationship between body size and nest area has been shown before (Tuomainen and Candolin, 2013).

Although UVB$_{enhanced}$ males in the present study were smaller they were not significantly different in sexual attractiveness (orange-red throat coloration) compared to UVB$_{normal}$ males. Differential investment in pre- and post-mating traits may have enabled

sticklebacks of the UVB_{enhanced} group to compensate potential limitations on pre-mating traits at the expense of functional fertility. Thus, the honesty of the pre-mating trait, i.e. intensity of red breeding coloration, could not be maintained when being exposed to stressful environmental conditions during growth. However, as males of the UVB_{enhanced} treatment still had motile sperm and thus were probably fertile, preserving nuptial coloration seems to be beneficial as it enhances access to potential matings. To what extent a shifted allocation ultimately can compensate negative effects like impaired growth under natural conditions, considering competition for food and territories, needs further research. Additionally, studies on the interactions of UV and other stressors (e.g. temperature: Jokinen *et al.*, 2011) or nongenetic inheritance mechanisms such as transgenerational plasticity (Shama and Wegner, 2014) may help to elucidate the ultimate consequences of climate change for the direct fitness of aquatic organisms. We provide experimental evidence that increasing levels of ambient UVB as environmental stress factor affect the investment in pre- and post-mating fitness-relevant traits suggesting that phenotypic responses are of importance in mitigating deleterious effects on post-mating sexual traits, potentially at the cost of sexual attractiveness.

Acknowledgements

Chapter IV

Long-term UVB exposure promotes predator-inspection behaviour in a fish

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Note: This chapter is written in British English according to the published version.

Chapter IV

Long-term UVB exposure promotes predator-inspection behaviour in a fish

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Abstract

Ultraviolet-B radiation (UVB) reaching the earth's surface has increased due to human-caused stratospheric ozone depletion. Whereas the harmful effects of UVB on aquatic organisms are well studied at the molecular and cellular level, recent studies have also begun to address behavioural changes caused by sublethal amounts of UVB. However, the behavioural consequences of long-term exposure to ecologically relevant UVB levels over several life stages are virtually unknown, particularly with regard to predator–prey behaviour. We found increased predator-inspection behaviour together with a smaller body length in three-spined sticklebacks (*Gasterosteus aculeatus*) after fish were exposed for about seven months to natural sunlight conditions with enhanced UVB, compared with full siblings exposed to natural sunlight only. The observed change in antipredator behaviour may reflect a direct behavioural response mediated through UVB-induced oxidative stress during development. Alternatively, the smaller body size in UVB-exposed fish may result in an increased inspection effort allowing them to spend more time foraging. Our findings suggest that, within the scope of environmental change, UVB radiation constitutes an important stress factor by eliciting behavioural responses that influence crucial ecological processes, such as predator–prey interactions.

Introduction

Ultraviolet radiation, and especially short-wavelength ultraviolet-B radiation (280–320 nm), constitutes an important environmental factor that negatively affects organisms (e.g. Häder *et al.*, 2011). UVB exposure of organisms living in aquatic habitats is expected to be influenced by climate change, particularly through recent and ongoing stratospheric ozone depletion, alterations in cloud cover and acidification (Häder *et al.*, 2011). UVB radiation causes direct damage to DNA and has indirect effects at molecular and cellular levels by inducing the

formation of reactive oxygen species (ROS) (Dahms and Lee, 2010). Furthermore, UVB affects development, fecundity and physiology at the whole-organism level with potentially far-reaching ecological consequences (e.g. Häder *et al.*, 2011; Vitt *et al.*, 2017a).

In fishes, in addition to physiological responses to increased UVB levels (Pulgar *et al.*, 2017), recent studies confirmed that UV can influence behaviours involved in predator–prey interactions in terms of a reduced escape performance in prey fish (Fukunishi *et al.*, 2012), a decrease in prey detection (Fukunishi *et al.*, 2013a), as well as alterations in locomotion (Seebacher *et al.*, 2016) and habitat selection (Kelly and Bothwell, 2002). However, these laboratory studies have focused mainly on consequences either of an acute direct UV exposure (e.g. Kelly and Bothwell, 2002; Pulgar *et al.*, 2017) or on effects following medium-term UVB exposure at larval or adult stages (Fukunishi *et al.*, 2012; Fukunishi *et al.*, 2013a; Seebacher *et al.*, 2016). In comparison, it is unknown how long-term exposure to ecologically relevant UVB levels shapes behaviours that play a role in a predation context, although this is necessary for understanding the ecological consequences of chronic environmental stress for predator–prey interactions.

When threatened by predators, behavioural responses of prey individuals may include direct predator avoidance or antipredator behaviour (Lima, 2002). A widespread, but seemingly counterintuitive form of antipredator behaviour is predator inspection, where one or more potential prey individuals approach a predator while benefiting from gaining information about the predatory threat (e.g. Frommen *et al.*, 2009).

In our study species, the three-spined stickleback (*Gasterosteus aculeatus*), predator-inspection behaviour is a well-studied phenomenon in the context of behavioural predator–prey interactions (e.g. McGhee *et al.*, 2013). Furthermore, sticklebacks typically inhabit shallow waters where UVB radiation constitutes a significant environmental stressor (Vitt *et al.*, 2017a). Taken together, the aim of the present study was to examine whether long-term exposure to increased, but naturally occurring UVB levels, for several months during the period of major growth, affects predator-inspection behaviour in sticklebacks.

Material and Methods

Study animals and treatments

Sticklebacks used in this study were laboratory-bred offspring of fish caught from a large anadromous population in Texel, the Netherlands. On 2 September 2015, at an age of three months, 40 fish from each of 28 full-sib families were split into two equally sized groups and

transferred to 56 enclosures (20 l) installed in four large (2500 l) outdoor tanks. All fish were fed on six days a week *ad libitum* with defrosted chironomid larvae.

One group was exposed to enhanced ambient UVB radiation (UVB$_{enhanced}$) while the other group received natural sunlight (UVB$_{normal}$). UVB radiation was applied for 2 h daily for UVB-summer conditions (7 September 2015 – 18 November 2015) and reduced to 1h daily for UVB-winter conditions (19 November 2015 – 14 April 2016). Mean irradiance and daily doses of UVA and UVB are given in table 1. The maximum UVB intensity measured under UVB$_{enhanced}$ conditions reached 0.51 W m^{-2} and was within levels measured in March 2013 during midday under sunny weather conditions at 10 cm water depth in the natural habitat of our study population on the island of Texel (53°114' N, 4°898' E) peaking at 0.63 W m^{-2}. See the online supplementary material for methodological details.

For the behavioural experiments, one randomly chosen stickleback out of each enclosure was used (N$_{UVB-normal}$ = 28, N$_{UVB-enhanced}$ = 28). Sixteen rainbow trout *Oncorhynchus mykiss* (standard length (SL) 15.3 ± 1.4 cm) served as potential predators and each trout was used for the maximum of one experimental trial per day.

Table 1: Mean irradiance during midday (W m^{-2}) and daily dose (kJ m^{-2}) of ultraviolet A (UVA) and ultraviolet B (UVB) radiation used in the exposure treatments.

Measurement	UVB-winter conditions				UVB-summer conditions			
	UV$_{enhanced}$		UV$_{normal}$		UV$_{enhanced}$		UV$_{normal}$	
Spectral range	UVA	UVB	UVA	UVB	UVA	UVB	UVA	UVB
Mean irradiance - midday (W m^{-2})	2.14	0.36	2.04	0.11	3.68	0.47	3.31	0.27
Daily dose (kJ m^{-2})	45.58	2.36	45.20	1.46	85.23	5.85	82.42	4.27

Predator-inspection experiments

From 30 March 2016 to 14 April 2016, at the age of 10.5 ± 0.5 months, fish were individually tested in an experimental tank (100 x 45 x 35 cm, water level 16.5 cm) consisting of a predator compartment (25 x 45 cm), separated by a fixed sheet of perforated clear plexiglas, an experimental compartment (60 x 45 cm) and a start zone (15 x 45 cm). The inspection zone was located within the experimental compartment, next to the predator compartment (figure 1, see online supplementary material for details).

Before each experiment, a transparent partition between the start zone and the experimental compartment and an opaque partition between the inspection zone and the predator compartment were lowered. For the experiments, a trout was placed in the predator compartment and a test fish was introduced in the start zone. After 10 min of acclimation time both partitions were lifted for 30 min of trial time so that the stickleback could enter the experimental compartment (figure 1). Trials were filmed from above using a webcam connected to a laptop. After each experiment, the standard length (SL) of the test fish was measured and the first dorsal spine was clipped off and used for molecular genetic sexing (for methods, see Bakker *et al.*, 2017).
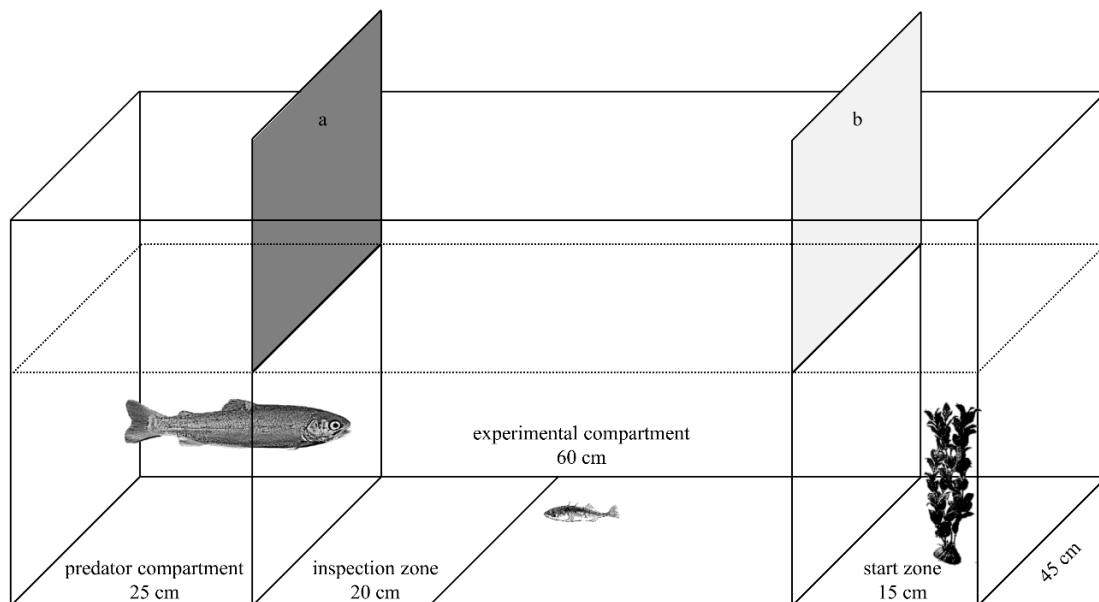

Fig. 1: The experimental tank with the predator compartment on the left, the experimental compartment in the middle, and the start zone with an artificial plant on the right. The inspection zone is located next to the predator compartment, within the experimental compartment. An opaque partition (a) and a transparent partition (b) were lowered during the acclimation phase and lifted for the experimental phase.

Data analyses

The latency of the test fish to initially leave the start zone and the absolute inspection time (IT_{abs} = absolute time in inspection zone) (followingBrown and Morgan, 2015) were quantified using the video recordings. Furthermore, we calculated the relative inspection time ($IT_{rel} = IT_{abs}$ / time after leaving start zone) to control for inter-individual differences in the latency to leave the start zone. Moreover, the numbers of visits to the start zone and to the inspection zone were used to calculate absolute activity ($activity_{abs}$ = visits to start zone + visits to inspection zone) and relative activity ($activity_{rel} = activity_{abs}$ / time after leaving start zone). Data were analysed using R v. 3.3.0 statistical package (R Core Team, 2016). Shapiro–Wilk tests were performed to test for normality. Except for SL, all variables were Box–Cox transformed to meet the assumptions of normality. Linear mixed-effects models (lme) were applied using the 'lmer' function in the 'lme4' library. Hierarchical random effects were used by nesting family in outdoor tank. A backward stepwise model reduction was conducted by removing the explanatory variables in the order of statistical relevance (e.g. Engqvist, 2005). Tests of significance were based on likelihood-ratio tests.

Results

Sticklebacks from the UVB_{normal}-group were, independent of sex, significantly larger compared with fish from the $UVB_{enhanced}$-group (lme, χ^2 = 22.162, p < 0.001) and, independent of treatment, females (N = 25: $N_{UVB-enhanced}$ = 11, $N_{UVB-normal}$ = 14) were significantly larger than males (N = 31: $N_{UVB-enhanced}$ = 17, $N_{UVB-normal}$ = 14) (lme, χ^2 = 18.092, p < 0.001).

All sticklebacks left the start zone within the experimental time (median: 20.50 s; interquartile range: 5.00–56.25 s) and showed characteristic predator-inspection behaviour in terms of repeated sequences of approaching the predator, pausing, turning and moving back to the opposite side during all trials. Trout were active and orientated towards the test fish most of the time. Sticklebacks from the $UVB_{enhanced}$-group showed on average a significantly higher absolute (IT_{abs}: lme, χ^2 = 5.538, p = 0.019; figure 2) as well as relative inspection time (IT_{rel}: lme, χ^2 = 5.213, p = 0.022) and thereby their predator-inspection behaviour was improved compared with UVB_{normal}-fish. Absolute and relative activity were not significantly different between treatments ($activity_{abs}$: lme, χ^2 = 0.025, p = 0.875; $activity_{rel}$: lme, χ^2 = 0.223, p = 0.637). Moreover, there was no significant difference between treatments with regard to the latency to leave the start zone (lme, χ^2 = 2.495, p = 0.114). See electronic supplementary material (table S1) for detailed results.
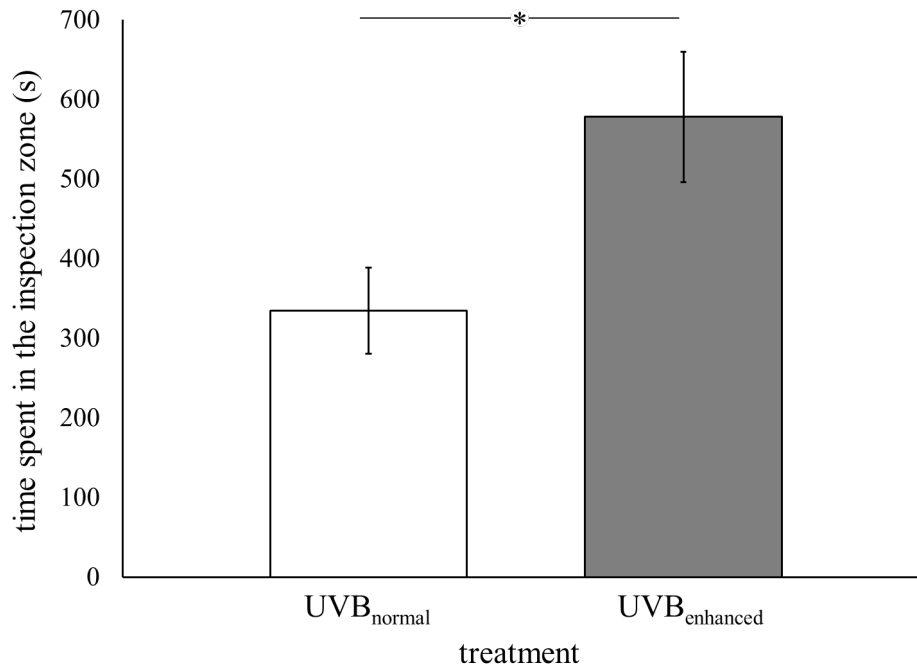
Fig. 2: Absolute times (mean ± s.e.m.) sticklebacks from the UVB$_{normal}$ (white) and UVB$_{enhanced}$ (grey) treatments spent in the inspection zone. Non-transformed data are presented for visual purposes only. * $p < 0.05$.

Discussion

Three-spined sticklebacks showed an increased predator inspection as a consequence of prolonged UVB exposure. In addition, UVB$_{enhanced}$-fish were smaller in size (SL), as has also been shown in a recent study dealing with UVB stress in sticklebacks (Vitt *et al.*, 2017a). This is possibly a result of resource allocation to repair mechanisms of UVB-induced ROS-mediated photodamage at the expense of growth (Olson and Mitchell, 2006).

Although predator-inspection behaviour is risky (Milinski *et al.*, 1997) and smaller fish are subject to a higher predation risk (Krause *et al.*, 1998), smaller UVB$_{enhanced}$-fish showed an increased inspection compared with larger UVB$_{normal}$-fish. It can be assumed that UVB-exposed sticklebacks face higher physiological costs, resulting in increased nutritional requirements. Consequently, these individuals may gain a foraging benefit through an increased predator inspection (Godin and Sproul, 1988). Moreover, it is important for physically constrained individuals to gather detailed information about a predator, as had been shown for gravid female sticklebacks (Frommen *et al.*, 2009). Furthermore, the increased inspection could be based on a demonstration of alertness or flight abilities in accordance to the 'attack deterrence hypothesis' (Hasson, 1991) with smaller sticklebacks being potentially more manoeuvrable. As long as prey individuals benefit from an increased inspection behaviour by gaining more information about a potential predator, they should be able to compensate for their UVB-

induced physical disadvantage. However, the net benefit should be outweighed by the costs of an increased mortality risk when the amount of predator-inspection behaviour is very high. Further investigations are needed to elucidate whether UVB-stressed prey individuals ultimately suffer from an increased mortality or not.

Independent of the aforementioned effects of UVB exposition on body size and possibly related behavioural responses, an enhanced formation of ROS may be responsible for the behavioural changes in UVB$_{enhanced}$-fish. Seebacher *et al.* (2016) found an increase in ROS-induced damage in UVB-exposed zebrafish (*Danio rerio*) together with a reduced swimming performance at high temperatures compared with fish from a control group. By contrast, activity levels were not different between treatment groups in the present study so that comparable ROS-mediated effects on swimming performance can largely be ruled out. Nevertheless, further research is required to identify the mechanisms and pathways underlying UVB-caused alterations in physiology and behaviour (e.g. Ghanizadeh-Kazerouni *et al.*, 2017).

In summary, long-term exposure to increased but natural levels of UVB radiation is capable of affecting predator–prey interactions through alterations in predator-inspection behaviour in sticklebacks. Short-term UVB exposure was found to affect predator–prey interactions, e.g. through impaired escape performance, as demonstrated in cod larvae (*Gadus morhua*) threatened by two-spotted gobies (*Gobiusculus flavescens*) (Fukunishi *et al.*, 2012). In comparison, our findings demonstrate for the first time to our knowledge that long-term exposure to even lower, ecologically relevant UVB levels influences antipredator behaviour in a fish. Given that UVB radiation is abundant in shallow water environments, UVB-mediated changes in ecological processes such as predator–prey interactions are likely to have important consequences for population and community dynamics in these ecosystems.

Ethics

Experiments complied with the current laws of Germany and were approved by the regional office for nature, environment, and consumer protection North-Rhine Westphalia (LANUV NRW, reference number 84-02.04.2015.A580).

Acknowledgements

Supplementary material

Experimental Subjects and holding conditions
Twenty-eight full-sib families of laboratory-bred sticklebacks were split into two groups of 20 individuals each (1120 sticklebacks in total) and transferred into 56 enclosures (39 × 28 × 28 cm, water level 20 cm), placed into four 2500 l outdoor tanks (14 enclosures / outdoor tank). From 07 September 2015 on, one group of each family was exposed to enhanced ambient UVB radiation for two hours from 11 a.m. to 1 p.m., provided by UVB lamps (G8T5E, 8W, Sankyo Denki, Japan) installed 10 cm above the water surface whereas for the other group opaque plastic strips were installed instead of the lamps at the same position to provide similar shade conditions. UVB-winter conditions, from 19 November 2015 to 14 April 2016, were created by reducing the irradiation time to one hour (11 a.m. to 12 midday). During the experimental phase, UVR was logged by a UV data logger (UVA / UVB, UV Microlog, Scitec Instruments, Bradford on Avon, GB). For the behavioural experiments, one randomly chosen stickleback out of each tank was used (N = 56) whereas remaining fish served for another study (S. Vitt et al., unpublished data).

Trout were kept in two large outdoor tanks (750 l) with air ventilation and a constant supply of tap water at a flow rate of $3 \, l \, min^{-1}$ and fed with commercial trout pellets six days a week.

Experimental setup
The experimental tank (100 cm x 45 cm x 35 cm) consisted of a predator compartment (25 cm x 45 cm) and a test fish compartment (75 cm x 45 cm). The predator compartment was separated by a fixed sheet of perforated clear plexiglas and a removable sheet of opaque plastic, respectively. The test fish compartment was divided by a removable, perforated clear plexiglas sheet into a start zone (15 cm x 45 cm) and the experimental compartment (60 cm x 45 cm). The inspection zone (20 cm x 45 cm) was located next to the predator compartment, within the experimental compartment. A plastic plant in the middle of the start zone served as a hiding place and a webcam (QuickCam Pro 9000, Logitech) attached to a laptop (ThinkPad T60, Lenovo) was mounted above the tank. The aquarium was illuminated by two fluorescent lamps (True Light, Natural Daylight 5500, 36 W, Aura Light) at 43 cm distance from the water surface. The whole set-up was surrounded by a black curtain and both removable sheets could be lifted from outside the curtain by a string, using a pulley system. Before each experiment, the aquarium was filled with one day-old tap water up to a level of 16.5 cm.

Irradiance Measurement in the field

Measurements in the field were made by using a spectrophotometer (AvaSpec 2048, Avantes, the Netherlands) equipped with a cosine corrector (CC-UV/VIS, Avantes, the Netherlands). Irradiance was calibrated against an Avantes NIST traceable application standard. For measurements, the irradiance probe was kept in a fixed position at 10 cm water depth. Fifteen single spectra were recorded within one measuring series using the software AvaSoft (Version 7.5, Avantes, the Netherlands) and transferred to Microsoft Excel (Microsoft Office 2007) to calculate a mean spectrum.

Linear mixed effects models

Table S1: All linear mixed effects models (lme) calculated. In the lmes regarding standard length (SL) either sex or treatment were taken out of the model because both significantly influenced the SL. Time until leaving the start zone (latency), activity and absolute time spent in the inspection zone were used as dependent variables. Treatment, sex and SL were used as explanatory variables. Hierarchical random effects were used in each model by nesting family within outdoor tank as random factors.

Dependent Variables	Explanatory variable									Random factors
	Treatment			Sex			SL			
	χ^2	Δd.f.	p	χ^2	Δd.f.	p	χ^2	Δd.f.	p	
SL	22.162	1	**< 0.001**	18.092	1	**< 0.001**	-	-	-	Outdoor tank Family
Latency	2.495	1	0.114	1.761	1	0.185	0.130	1	0.718	Outdoor tank Family
Activity	0.043	1	0.836	2.321	1	0.128	0.060	1	0.806	Outdoor tank Family
IPI	5.594	1	**0.018**	0.071	1	0.790	0.888	1	0.346	Outdoor tank Family
Start zone	5.216	1	**0.022**	0.002	1	0.966	0.098	1	0.148	Outdoor tank Family
Zone one	5.643	1	**0.018**	0.083	1	0.773	4.928	1	**0.026**	Outdoor tank Family
Zone two	0.232	1	0.630	0.033	1	0.856	4.146	1	**0.042**	Outdoor tank Family
Zone three	5.404	1	**0.020**	0.111	1	0.739	0.361	1	0.548	Outdoor tank Family

General discussion

The direct effects of a changing environment in terms of climate change are the focus of countless studies investigating effects in various taxa ranging from microbial communities (reviewed in Singh *et al.*, 2010) to plants (reviewed in Gray and Brady, 2016), insects (reviewed in Arribas *et al.*, 2017) and vertebrates (e.g. Carozza *et al.*, 2019; Valenzuela *et al.*, 2019). In most empirical studies, specific physiological responses of organisms exposed to an environmental stressor, e.g. elevated temperature or UVR, were measured or trophic interactions among different taxa, e.g. plants, insects and vertebrates, were studied (reviewed in Renner and Zohner, 2018). Alternatively, ecological consequences of environmental stressors are often evaluated, which are based on observations and measurements under natural conditions, but do not use experimental approaches.

In my thesis, I have combined the advantages of using natural conditions together with a controlled experimental design. To do this, I used semi-natural conditions including natural sunlight to investigate the effects of enhanced, but ecologically relevant ambient UVB radiation on a small teleost fish species, the three-spined stickleback (*Gasterosteus aculeatus*). In this species, both sexes use UV signals in various contexts, e.g. during intrasexual communication (e.g. Rick *et al.*, 2006; Hiermes *et al.*, 2015c; Hiermes *et al.*, 2016), foraging (Boulcott and Braithwaite, 2005; Modarressie and Bakker, 2007; Rick *et al.*, 2012) and habitat preference (Rick and Bakker, 2010). Thus, the avoidance of UVR, e.g. by migration to areas without or with reduced ambient UVR, like deeper waters or shady areas, would prevent the perception of UV signals. Consequently, sticklebacks are, at least partially, reliant on ambient UVR and therefore a suitable species for examining costs of increasing ambient UVR.

I used different experimental approaches to gain comprehensive knowledge on the consequences of the abiotic stressor UVB on multiple life-history traits. On the one hand, I exposed sticklebacks to chronic UVB radiation for different periods, ranging from about two months (*chapter I* and *chapter II*) to seven months (*chapter IV*) and nine months (*chapter III*). On the other hand, I investigated different topics of the biology of sticklebacks. The focus of *chapter I* was to examine the immunological consequences of UVB for the immune system in non-reproductive sticklebacks of both sexes. In *chapter II* and *chapter III*, the differential investment in the expression of pre- and post-mating sexually selected traits was studied by exposing adult, reproductively active male sticklebacks (*chapter II*) and sticklebacks during their major growth phase (*chapter III*) to elevated levels of ambient UVB. In *chapter IV*, I

examined the behavioral consequences of long-term exposure to ecologically relevant UVB levels with regard to predator-prey behavior.

UVB effects on growth and body condition

Subadult sticklebacks exposed to enhanced levels of ambient UVB for about two months showed reduced growth as well as negative effects on body condition compared to individuals exposed to natural solar radiation (*chapter I*). Moreover, sticklebacks exposed to similar amounts of UVB for seven (*chapter IV*) and nine months (*chapter III*), from early-life on, also showed reduced growth (*chapter III* and *chapter IV*) and gained less weight compared to their full-sibs raised under natural sunlight (*chapter III*). In the present thesis, individuals exposed to enhanced UVB and those exposed to natural sunlight received equal amounts of food and were kept under identical thermal conditions. Consequently, it is very likely that differences in growth, condition and weight are a result of UVB-mediated differential resource allocation towards other processes. Correspondingly, impaired growth in UVB-exposed fish was also shown in juvenile Atlantic salmon (*Salmo salar*) (Jokinen *et al.*, 2008) and larval anchovy (*Egradulis mordax*) (Hunter *et al.*, 1981). UV and especially the higher energetic UVB radiation have a broad range of adverse effects on aquatic organisms, ranging from an impaired immune function (*chapter I*) and increased oxidative stress (e.g. Häder *et al.*, 2015; Seebacher *et al.*, 2016) to the destruction of lipids, nucleic acids, proteins (Bancroft *et al.*, 2007) and membranes (Lesser *et al.*, 2001). Such diverse damages necessarily induce costly repair mechanisms (e.g. Metcalfe and Alonso-Alvarez, 2010; Ling *et al.*, 2017). For instance, if an organism's antioxidant capacity is not able to reduce harmful effects of ROS, diverse components of animal performance can be affected (reviewed in Monaghan, 2007). Thus, trade-offs regarding the investment in different life-history components are supposed to be pronounced under stressful conditions (e.g. Alonso-Alvarez *et al.*, 2006) and are expected to result in a differential resource allocation between opposing physiological requirements (Monaghan *et al.*, 2009). Additionally, adverse effects on growth and body condition may be reinforced by an impaired digestion caused by UV (Ylönen *et al.*, 2004; García-Huidobro *et al.*, 2017). Whereas the exact physiological effects of UVB on the digestive system are unclear, Ylönen *et al.* (2004) discuss either a decreased mitochondrial capacity supplying energy to the digestive system or a disturbed blood circulation in the digestive system as potential explanations. The absent effects on growth and body condition in *chapter II* of this thesis could likely be explained by the fact

that in this experiment adult individuals were used and potential effects on growth and condition may be less pronounced as in juvenile or subadult individuals.

Taken together, chronic exposure to enhanced ambient UVB has serious effects on growth and physical condition in three-spined sticklebacks and has the potential to disturb metabolic processes. However, the exact physiological mechanisms need to be addressed in further research.

Immunomodulatory effects of UVB

Fishes, representing a heterogenous group of organisms, are described to have a basal position in vertebrate phylogeny, which makes them attractive as model organisms in studies investigating immunological processes. In teleost fishes, the immune system basically consists of two components, the innate and the adaptive immunity (Uribe *et al.*, 2011). The non-specific innate immunity represents a fundamental defense mechanism, including physical barriers, cellular and humoral components (Magnadóttir, 2006). The adaptive immunity, also known as acquired immunity, consists of specific immune-responses with lymphocytes as the key effector cells (Lieschke and Trede, 2009). The immune system of fishes is described as a crossroads between innate and adaptive immunological responses (Tort *et al.*, 2003; Rauta *et al.*, 2012).

Chapter I shows that enhanced ambient UVB has a strong impact on the immune system, i.e. the differential investment in the innate and adaptive immunity of three-spined sticklebacks. A lower SSI (splenosomatic index) together with a higher G/L ratio (granulocyte-to-lymphocyte ratio) in UVB-exposed individuals compared to the control group shows the serious effects of enhanced ambient UVB on immunological processes and describes a shifted investment towards the innate immunity. In fishes, the spleen represents a lymphoid organ which has a major role in the adaptive immunity as it includes macrophages, lymphocytes and melanomacrophages (Zapata *et al.*, 2006; Kurtz *et al.*, 2007) and is involved in cytokine production (Castro and Tafalla, 2015). Accordingly, the SSI is positively related to the activation of the adaptive immunity in juvenile Arctic charr (*Salvelinus alpinus*) infected with the chronic parasite *Diplostomum* spp. (Seppänen *et al.*, 2009). Thus, the lower SSI, independent of sex, in sticklebacks exposed to enhanced UVB reported in *chapter I* could be interpreted as a reduced adaptive immunity following UVB exposure. Independent of exposure treatment, male sticklebacks had smaller spleens which might potentially be caused by androgen-induced immunosuppression, e.g. as shown in birds (Møller *et al.*, 1998). Although individuals used in the experiment of *chapter I* were subadults, they were close to reaching the

reproductive phase and testosterone levels likely differed between sexes. Accordingly, pre-breeding aggression has been observed in three-spined stickleback males (Bakker, 1994) and testosterone levels were found to be associated with aggression in fishes (Li *et al.*, 2014). Thus, an androgen-induced reduced SSI in sticklebacks preceding the reproductive phase cannot be excluded.

A trade-off between innate and adaptive immunity is generally assumed (e.g. Norris and Evans, 2000), which could explain the higher G/L ratio of head kidneys in sticklebacks exposed to enhanced UVB (*chapter I*). Here, the ratio of granulocytes and lymphocytes of the head kidney was determined. The head kidney, analogous to the mammalian adrenal gland, is an important endocrine and haematopoietic-lymphoid organ in teleost fishes (Uribe *et al.*, 2011). Thus, measuring the ratio of head kidney granulocytes to lymphocytes is an often-applied method in studying the immune response of fishes (e.g. Kalbe and Kurtz, 2006; Scharsack *et al.*, 2007). Granulocytes are involved in the immediate, rather unspecific response of the innate immunity, e.g. following inflammatory processes (Hine, 1992). Lymphocytes represent a major part of the adaptive immune system and mediate highly specific immune responses (Flajnik and Kasahara, 2010). In *chapter I*, sticklebacks exposed to enhanced ambient UVB showed increased activity of the innate compared to the adaptive immune system (higher G/L ratio). During the experiments described in *chapter I*, no signs of skin alteration, e.g. sunburns, were detected. Nevertheless, UVB may have induced inflammatory responses (i.e. erythema) followed by corresponding immune responses. Accordingly, in juvenile rainbow trout UVB irradiation resulted in necrosis, epidermal sunburn cell formation (apoptosis) and declining mucous cells in the epidermis (Abedi *et al.*, 2015). Thus, in three-spined sticklebacks, enhanced ambient UVB may have caused inflammatory processes, followed by an increased activity of the innate compared to the adaptive immune system. I exposed individuals to enhanced UVB daily for two months. Such chronic exposure may have caused a permanent activation of the innate immune system, likely followed by its permanent intensification and, consequently, a shifted G/L ratio of head kidney cells towards granulocytes. During the experiments, sticklebacks were kept in a closed tank system without parasites or contagious diseases, which was confirmed during the dissections. Thus, a relative increase in activation of the innate immune system (higher G/L ratio) is likely beneficial in the absence of pathogens and when being confronted with an environmental stressor which has the potential to induce inflammatory processes. However, under natural conditions, sticklebacks are exposed to a variety of parasites, e.g. the eye fluke (*Diplostomum pseudospathaceum*), or the cestode (*Schistocephalus solidus*) which are antagonized by the adaptive immunity (Piecyk *et al.*, 2019). Hence, it can be assumed

that exposure to UVB will impair the efficacy of the adaptive immune response to pathogens, resulting in more severe infections or conversion of subclinical infections into symptomatic ones (Lawrence *et al.*, 2020). The connection between innate and adaptive immunity is well studied (reviewed in Tort *et al.*, 2003; Uribe *et al.*, 2011) and of great importance as it is linked to consequences for the whole organism. Particularly in terms of a changing environment with increasing UVB levels and potential interactive effects of further abiotic and biotic factors, a differentiated investment in innate and adaptive immunity is likely very costly and may result in a reduced fitness (e.g. Ahmed *et al.*, 2002; Graham *et al.*, 2011). In *chapter I*, I revealed serious immunomodulatory effects of an exposure to enhanced ambient UVB levels and showed that even when resource availability was sufficient and additional stressors, e.g. parasites or predation risk excluded, individuals could not compensate for the adverse UVB effects on immunity. Ultimately, such effects can be expected to appear even stronger under natural conditions, which should be addressed in further studies. For example, transgenerational plastic responses of immune functions to enhanced UVB are worth investigating and were already observed in pipefish of the species *Syngnathus typhle* following increased temperature (Roth and Landis, 2017). Furthermore, climate change affects parasites and infectious diseases in aquatic ecosystems (Marcogliese, 2008; Marcogliese, 2016) followed by challenges for the adaptive immunity (Lõhmus and Björklund, 2015), which could alter the shifted investment in immunity described in this thesis.

UVB effects on resource allocation to pre- and post-mating traits

In male three-spined sticklebacks, the investment in pre- and post-mating sexually selected traits determines reproductive success. At the pre-mating level, sexual attractiveness (expression of ornaments) is a major factor in gaining access to mating partners in terms of inter- and intrasexual selection. At the post-mating level, sperm-related traits significantly determine an individual's reproductive success. When resources are limited and/or stress is present, the investment in pre- and post-mating traits could be affected differently. The phenotype-linked fertility hypothesis (PLFH) by Sheldon (1994) suggests that the honesty of sexual ornamentation is preserved by a link with fertility. In accordance, a study examining datasets describing plumage and sperm morphological traits for 278 passerine bird species found a significant positive relationship between sperm midpiece length and male plumage elaboration and sexual dichromatism (Durrant *et al.*, 2020). Assuming that longer midpieces make sperm more competitive and successful at fertilization (Snook, 2005), this study provides

strong support for the PLFH. However, next to further studies supporting the PLFH (e.g. Locatello *et al.*, 2006; Hosken *et al.*, 2008; Mehlis *et al.*, 2013), a variety of studies showed no support for the PLFH, i.e. no positive relationships between pre- and post-mating traits or even negative relationships (reviewed in Mautz *et al.*, 2013). Such negative relationships between pre- and post-mating traits are predicted by the sperm competition theory (SCT) by Parker (1990). The SCT proposes that males compensate for limited access to matings, e.g. through less pronounced sexual ornaments, by increasing the investment in spermatogenesis at the expense of pre-mating traits. The inconsistency in studies examining the investment in pre- and post-mating traits, i.e. support for PLFH or SCT, is likely based on the effect of additional life-history, ecological, and mating-system variables which significantly determine such resource allocation (reviewed in Simmons *et al.*, 2017).

*C*hapter *II* and *chapter III* deal with the impact of enhanced ambient UVB on sexually selected pre- and post-mating traits. Pre-mating traits were studied by quantifying the expression of the carotenoid-based sexual ornamentation of male three-spined sticklebacks (*chapter II* and *chapter III*). At the post-mating level, multiple sperm-related traits were examined. In *chapter II* and *chapter III*, testes mass, the number of sperm and sperm movement were used to determine the investment in post-mating traits. In *chapter II*, sperm morphology was also analyzed and in *chapter III*, testes brightness, i.e. melanization, and testicular total antioxidant capacity was measured. Furthermore, *chapter III* included nest-building traits (timespan for building and nest area).

A major part of the present thesis, including *chapter II* and *chapter III*, deals with differential resource allocation to pre- and post-mating traits in male three-spined sticklebacks that were exposed to enhanced, but ecologically relevant ambient UVB during different life stages. In contrast to most studies on biological UVB effects, which mainly applied single doses or used shorter exposure periods in the laboratory (summarized in Lawrence *et al.*, 2020), I exposed individuals chronically to enhanced ambient UVB in addition to natural sunlight in a semi-natural setup. On the one hand, this thesis aimed to explore physiological responses to an environmental stressor in adult male sticklebacks which were reproductively active during exposure. On the other hand, effects of exposure during the major growth phase, from early-life on, were examined. Therefore, maintenance of already expressed traits as well a potential shifted allocation preceding the development of sexually selected traits were addressed and put into context. Both exposure for about two (*chapter II*) and nine (*chapter II*) months are ecologically relevant and represent naturally occurring conditions, as extreme weather events lasting for shorter periods as well as persistent environmental changes are well documented.

For instance, short-term changes were shown during the European heat wave in the summer 2003, which is described as one of the most severe natural disasters in recent European history (Mitchell *et al.*, 2016) and included an intense low-ozone episode over Scandinavia and the North Sea (Orsolini and Nikulin, 2006). During low-ozone episodes, the lowering of the ozone column together with cloud-free anticyclonic conditions increase UV radiation reaching earth's surface (Schwarz *et al.*, 2018), which highlights the importance of studies examining short-dated changes of photic conditions. The frequency of extreme weather events is increasing worldwide (e.g. Mann *et al.*, 2017; Ummenhofer and Meehl, 2017) and has also been shown for Europe in particular (e.g. Kron *et al.*, 2019). Long-term changes of earth's climate were investigated in various studies (e.g. Butikofer *et al.*, 2019; Turner *et al.*, 2020) including changes in photic conditions (e.g. Bais *et al.*, 2019; Williamson *et al.*, 2019).

By comparing effects of exposure to enhanced levels of UVB on adult and growing individuals, I provide empirical evidence for adaptations to persistent or acute environmental stress. In *chapter II*, I demonstrated that enhanced ambient UVB negatively affects post-mating traits in adult male sticklebacks, expressed by a lower testes mass, a lower sperm number and shorter sperm. In contrast, UVB-exposed individuals showed no significant differences in the measured pre-mating traits, i.e. red intensity or hue of the nuptial coloration. Testis mass and sperm number are linked in sticklebacks and a reduced sperm number is supposed to result in impaired competitiveness during sperm competition and reduced fertilization success (Stockley *et al.*, 1997). Accordingly, Mehlis and Bakker (2014) showed that sperm number in sticklebacks is correlated with the fertilization rate in competitive and non-competitive contexts. The amount of sperm during the reproductive phase is described to be limited as spermatogenesis in sticklebacks is mainly restricted during this phase (Borg, 1982). However, Sokolowska and Kulczykowska (2006) showed that, at least in two populations of three-spined sticklebacks, spermatogenesis also takes place in reproductively active males. Consequently, the observed impaired sperm traits, i.e. shorter sperm and lower numbers in individuals exposed to enhanced ambient UVB, as described in *chapter II*, may be explained by deleterious effects on spermatogenesis. Phenotypically plastic modifications of sperm quality have been shown before, e.g. in Gouldian finches (*Erythrura gouldiae*) which produce longer sperm-midpieces in highly competitive social environments (Immler *et al.*, 2010). Competition was also shown to induce phenotypically plastic changes in zebrafish (*Danio rerio*) with males confronted with rival males producing larger midpieces and flagella of sperm which showed better swimming abilities and were more competitive (Silva *et al.*, 2019). Shorter sperm were observed in zebra finches (*Taeniopygia guttata*) as a phenotypic plastic response to oxidative stress (Tomášek *et*

al., 2017). Next to potential effects of UVB-induced ROS on sperm morphology, formation of ROS may also have caused the described differences in sperm number (*chapter II*). Accordingly, ROS were shown to be a major cause of sperm damage (Zan-Bar *et al.*, 2005) and e.g. cause increased lipid peroxidation in sea urchin sperm resulting in impaired fertilization success (Lu and Wu, 2005b).

Chapter II neither provides support for the PLFH (Sheldon, 1994) nor for the SCT (Parker, 1990) as I did not find any link between pre- and post-mating traits, which is likely caused by the absent effects on male nuptial coloration. Interestingly, Mehlis *et al.* (2015) found a positive relationship between the expression of pre- and post-mating traits under no food limitation, whereas resource limitation resulted in a negative relationship. Similar effects are described by Pike *et al.* (2007). Absent effects on coloration in *chapter II* seem to contradict a previous study by Rick *et al.* (2014) who found adverse effects of enhanced UVA radiation on male breeding coloration in sticklebacks after exposure to enhanced UVA in a laboratory study. Absent effects of UVB on color traits may be caused by the greater ability of UVA to reach deeper layers of the skin compared to UVB (Herrling *et al.*, 2006) and thereby having a stronger effect on pigment cells located within the dermis of teleost fishes (Hawkes, 1974). Alternatively, UVB effects on coloration may be masked by reduced coloration in both exposure treatments as response to a potential risk of predation. Accordingly, a reduced coloration has been observed in sticklebacks facing the potential threat of predation by birds in order to appear less conspicuous (Candolin, 1998).

Chapter II describes the serious effects on post-mating traits in sticklebacks and raises the question of how these negative consequences may be counteracted when UVB stress is present preceding the development of those traits. Thus, individuals used in the experiments of *chapter III* were exposed to enhanced ambient UVB from early life on, for about nine months, whereas the control group, consisting of full-sib brothers, received natural sunlight. In accordance with two months of exposure (*chapter II*), nine months of exposure to enhanced ambient UVB (*chapter III*) also had no significant effect on the measured color traits, contrasting previously observed UVA effects on coloration (Rick *et al.*, 2014). Absent effects on ornamentation may also be caused by a lower skin penetration of UVB compared to UVA.

At the reproductive peak, a trade-off between the investment in a pre-mating (color intensity) and a post-mating (sperm number) trait was found. In addition, despite being smaller and lighter, males raised under enhanced levels of UVB built their nests significantly faster than their brothers raised under solar radiation. Furthermore, the relationship between standard length and nest area was negative in UVB-stressed individuals whereas it was *vice versa* in the

control group. Interestingly, none of the significant effects on post-mating traits described in *chapter II* were observed in *chapter III* which also contradicts a study showing negative effects of UV exposure on sperm of blue tilapia (*Oreochromis aureus*), expressed by impaired motility and fertility of the sperm (Zan-Bar *et al.*, 2005). The persistent exposure to enhanced levels of UVB from early-life on likely triggered phenotypic plastic responses in male three-spined sticklebacks. Such responses may have compensated the adverse effects which were observed in individuals of *chapter II*, that were already adult and reproductively active. However, such compensation should be linked to impairments of other physiological traits and may have contributed to the reduced growth in UVB-stressed sticklebacks. Nevertheless, examination of the relationships between growth and post-mating traits did not reveal an investment in post-mating traits at the expense of growth, at least regarding the measured traits.

Spermatogenesis is a fitness-relevant process and may be involved in compensatory mechanisms which could explain the absence of significant effects at the post-mating level. As mentioned before, spermatogenesis in sticklebacks mainly takes place from late autumn to early winter and the amount of sperm is restricted during the breeding season (Borg, 1982). Consequently, compensatory effects during spermatogenesis are constrained which may have caused the negative UVB effects on sperm described in *chapter II* and the negative UVA effects examined by Rick *et al.* (2014). In *chapter III*, UVB-stressed individuals may have reinforced spermatogenesis preceding the reproductive phase which could explain absent differences in sperm numbers described in *chapter II*.

Surprisingly, no differences between UVB-stressed sticklebacks and the control group regarding the total antioxidant capacity (TAC) of the testes were observed in *chapter III*. Increased oxidative stress as a result of UV exposure has been described multiple times in fishes, e.g. for Atlantic cod, which showed higher activities and protein concentrations of the antioxidant enzyme superoxide dismutase (Lesser *et al.*, 2001) or in the yellowtail clownfish (*Amphiprion clarkia*) with increased activities of antioxidant enzymes (Ryu *et al.*, 2019). As discussed for the absent effects on breeding coloration, individuals may have shifted resource allocation at the expense of other processes, e.g. immunity or growth. Correspondingly, no effects on testicular melanization were found. However, independent from exposure treatments, TAC correlated with testicular melanization, which is in line with studies showing testicular melanization being linked to protection of gonadal tissue against oxidative stress (Plonka *et al.*, 2009; Galván *et al.*, 2011).

A major aspect of the present thesis is UVB-induced effects on resource allocation in terms of a shifted investment in pre- and post-mating traits of three-spined sticklebacks. In

chapter II, I found no significant relationship between pre- and post-mating traits in adult males after exposure to enhanced UVB for two months. However, in *chapter III*, I found a positive, although not significant, association between the intensity of the red breeding coloration and sperm number in the control group, exposed to solar radiation, whereas a significant negative correlation was observed in UVB-stressed males, which resulted in a significant interaction term across both exposure groups. A positive relationship between sexual ornamentation and sperm-related traits (functional fertility) is compliant with the PLFH by Sheldon (1994) because the honesty of a pre-mating sexual selected traits (sexual ornament) is preserved by a link to fertility. This link is absent and even reversed in individuals raised under stressful conditions with enhanced ambient UVB. Stressed males could not maintain the positive relationship, i.e. the positive link to fertility, between a pre-mating (coloration) and a post-mating (sperm number) trait. Apparently, individuals raised under enhanced ambient UVB reacted with a shifted investment towards a pre-mating trait (red intensity of the breeding coloration), at the expense of a post-mating trait (sperm number). One explanation might be that stressed males maintained their sexual attractiveness as there were no significant differences in the expression of breeding coloration between both exposure groups. In sticklebacks, the expression of the nuptial coloration plays a major role during intrasexual competition and is linked to mating success (Bakker and Milinski, 1993). Consequently, resource allocation towards coloration can be assumed to be beneficial for male sticklebacks even when being associated with adverse effects on other processes. Thus, a low-carotenoid diet was followed by shifted allocation of carotenoids towards the expression of coloration at the expense of body carotenoids and resulted in negative fitness consequences, i.e. significantly fewer breeding cycles and a shorter lifespan (Pike *et al.*, 2007). In accordance, Kim and Velando (2020) showed that stickleback males that invested more in red coloration had higher levels of oxidative DNA damage in muscle, testis and sperm measured at the peak of the breeding season, revealing a trade-off between carotenoid-based ornamentation and oxidative damage.

Three-spined stickleback males raised under enhanced ambient UVB were smaller and built their nests, independent of body length, faster than the control group exposed to natural sunlight, whereas nest size did not differ significantly (*chapter III*). While the relation between body size and nest area tended to be positive for males exposed to natural sunlight, this relationship was reversed, but not significantly, for UVB-stressed males, resulting in a significant interaction term (*chapter III*). As mentioned before, stickleback nests can act as extended phenotype and play a role in mate acquisition (Barber *et al.*, 2001; Östlund-Nilsson and Holmlund, 2003). Consequently, stressed males invest more energy in a pre-mating

behavior, i.e. nest-building, than their brothers of the control group and may benefit from less construction time by enhancing the chance of gaining matings. Moreover, smaller males with nests of similar size as larger males may appear more attractive to females as it suggests a good physical condition. Taken together, sticklebacks raised under UVB stress seem to allocate resources toward a pre-mating trait (intensity of the breeding coloration) and thereby potentially increase their opportunities of gaining access to matings.

In *chapter III*, independent of exposure treatment, males with a more orange-shifted coloration had a higher sperm number, i.e. seem to invest more in a post-mating trait. A reduced availability of certain high-quality carotenoids, e.g. astaxanthin, was shown to result in more orange coloration (Pike *et al.*, 2011) which was also shown by Mehlis *et al.* (2015) for sticklebacks fed with normal red mosquito larvae (more orange) compared to larvae enriched with astaxanthin (more red). As the red hue in male sticklebacks is linked to quality (e.g. Bakker and Milinski, 1993; Wedekind *et al.*, 1998), orange-shifted coloration likely is less attractive and these males may compensate for reduced attractiveness with a promoted spermatogenesis. Accordingly, in fruit flies (*Drosophila melanogaster*) winners of male-male conflicts have a higher reproductive success compared to losers, which invest more into post-mating mechanisms, e.g. expressed by longer copulations, followed by an increased paternity share when they were the first males to mate (Filice and Dukas, 2019).

In summary, I showed that enhanced levels of ambient UVB during the reproductive phase in three-spined sticklebacks cause adverse effects at the post-mating level, whereas the pre-mating color traits did not differ from the individuals raised under natural sunlight. Long-term exposure, from early life on, was followed by phenotypic plastic responses in terms of a shifted resource allocation to a pre-mating trait which likely maintained sexual attractiveness. Such shifted investments into fitness-relevant traits are essential in dealing with a stressful environment and reveal the importance of phenotypic responses in mitigating adverse effects. To what extent such responses occur under natural conditions, including additional stressors, i.e. limited availability of food or the presence of pathogens, should be addressed in further studies. For instance, for Atlantic herring (*Clupea harengus*) it was shown that climate change affects survival by causing food web changes (Sswat *et al.*, 2018). In addition, synergistic interactions between enhanced UVB and other abiotic factors, e.g. elevated temperature or water acidification, have already been described (e.g. Cramp *et al.*, 2014), but require further research.

UVB effects on behavior

Enhanced ambient UVB causes a broad range of adverse effects on various physiological processes, ranging from growth to immunity and a shifted investment in pre- and post-mating traits which is dealt with in *chapters I–III*. Next to effects on physiology and immunity, UVB can also affect the behavioral level in animals. Thus, c*hapter IV* deals with the behavioral consequences of long-term exposure to ecologically relevant UVB levels with regard to predator-prey interactions. After seven months of exposure to enhanced ambient UVB, sticklebacks showed increased predator-inspection behavior compared to full-sibs exposed to solar radiation.

UVB-mediated changes in behaviors were the focus of several studies on multiple taxa. The phytophagous insect (*Caliothrips phaseoli*) showed less invasive behavior of the canopies together with reduced herbivory after UVB exposure (Mazza *et al.*, 1999). In the green peach aphid (*Myzus persicae*), the UVA/UVB ratio affected orientation and spatial distribution over time (Dáder *et al.*, 2017) and in sea urchins (*Strongylocentrotus intermedius*) a one-hour exposure to UVB radiation significantly reduced foraging behavior (Shi *et al.*, 2018). In vertebrates, it was shown for amphibians that UVB exposure caused a reduced response to chemical extracts from conspecifics and heterospecifics, i.e. decreased refuge behavior (Kats *et al.*, 2000). In fishes, UVA and UVB caused an altered shelter selection in the transitory fish, *Girella laevifrons* (Pulgar *et al.*, 2017), and in juvenile rainbow trout UVB led to increased swimming activity and restless behavior (Alemanni *et al.*, 2003). Next to such negative UV effects, UVB can also have positive effects on the behavior, e.g. in mice (*Mus musculus*) in terms of improved motor learning and object recognition memory (Zhu *et al.*, 2018).

Chapter IV deals with effects on predator-prey interactions between three-spined sticklebacks as prey and rainbow trout as predators. Predator inspection is a widespread behavior in fishes, where individuals gain information about the predatory threat by approaching a potential predator, which has been well-studied in sticklebacks (e.g. Frommen *et al.*, 2009; McGhee *et al.*, 2013). In *chapter IV*, I demonstrated that UVB-exposed individuals showed promoted inspection behavior despite being smaller than their full-sibs exposed to natural sunlight. Predator inspection is risky (Milinski *et al.*, 1997) and smaller fish face a higher predation risk (Krause *et al.*, 1998), which makes the UVB-caused behavioral changes described in *chapter IV* seem counterintuitive. The mentioned effects on physiological and immunological processes described in *chapters I–III* likely result in higher energetical needs of sticklebacks raised under enhanced levels of UVB, potentially increasing their nutritional

requirements. Thus, it might be more important for stressed individuals to gain information about the predatory threat, as this leads to advantages in foraging. Accordingly, sticklebacks parasitized by the cestode *Schistocephalus solidus*, which have a greater need for energy than uninfected fish, showed reduced escape behavior, reduced motionless behavior, started foraging in a food patch near the predator sooner and were more active within a food patch after being frightened by a model heron (Godin and Sproul, 1988). Moreover, for physically constrained sticklebacks, gaining information about a potential predatory threat is important for survival which may have contributed to the increased predator inspection of UVB-stressed and growth-restricted individuals. In accordance, gravidity was shown to promote predator inspection in sticklebacks (Frommen *et al.*, 2009). Alternatively, smaller sticklebacks may be more maneuverable and may demonstrate alertness or flight ability, which would support the 'attack deterrence hypothesis' by Hasson (1991). As long as those benefits are not outweighed by increased mortality, they may contribute to the compensation of the aforementioned UVB-induced higher physiological needs. Whether UVB-stressed sticklebacks ultimately suffer from increased mortality needs further investigation. In cod larvae, an increased predation by two-spotted gobies (*Gobiusculus flavescens*) was observed after short-term exposure to UVB (Fukunishi *et al.*, 2012). However, in the mentioned study by Fukunishi *et al.* (2012) the predators were not stressed by UVB and it was shown in juveniles of the reef fish *Patagonotothen cornucola*, that individuals exposed to UVR took more time to detect and capture their prey (Valiñas and Walter Helbling, 2016).

The observed promoted predator inspection in UVB-stressed sticklebacks may also be linked to the release of stress hormones affecting the antipredator behavior. In accordance, Kats *et al.* (2000) showed that UVB exposure caused an increase in stress hormones which impaired the antipredator behavior of two amphibian species. After being exposed to UVB, toad (*Bufo boreas*) juveniles responded less to alarm cues compared to juveniles that had not been exposed and UVB-exposed frog tadpoles (*Rana cascadae*) did not decrease their activity in response to chemical cues from predators as much as their conspecifics that had not been exposed (Kats *et al.*, 2000). However, Lawrence *et al.* (2018a) found no relationship between elevated cortisol levels and behavior in the presence of a predator, i.e. shelter use and activity, in schoolmaster snapper (*Lutjanus apodus*) when confronted with lemon sharks (*Negaprion brevirostris*). Furthermore, experimental cortisol elevation did not mediate risk-taking and antipredator behavior, e.g. post-attack swimming duration, in pumpkinseed sunfish (*Lepomis gibbosus*) (Lawrence *et al.*, 2018b). However, higher cortisol levels were shown to increase the metabolic rate in pumpkinseed sunfish (Lawrence *et al.*, 2019) and gilthead sea bream (*Sparus aurata*)

(Aedo *et al.*, 2019), which may have contributed to the UVB-caused higher energetical needs followed by increased predator inspection as discussed above.

Taken together, long-term exposure to enhanced ambient UVB during growth has serious effects on the behavior of three-spined sticklebacks facing a predatory threat. Predator-prey interactions are a central factor in community dynamics and directly affect an individual's survival. Climate change, especially global warming, hypoxia and ocean acidification, can have a strong impact on physiology and behavior and has been shown to affect the kinematics of predator-prey interactions of fishes (reviewed in Domenici *et al.*, 2019). However, most studies showed that changing environments reduce the escape performance in fishes and explain such results as impaired muscle performance and/or negatively affected brain and sensory functions (Domenici *et al.*, 2019). Other studies examining UVB effects on predator-prey interactions in fishes used artificial conditions, e.g. single-exposure for 15 hours (Fukunishi *et al.*, 2012; Fukunishi *et al.*, 2013a) and focused on escape performance (Fukunishi *et al.*, 2012) or prey consumption (Fukunishi *et al.*, 2013a). To my knowledge, the effects described in *chapter IV* show for the first time that long-term exposure to ecologically relevant UVB levels influences antipredator behavior in a fish, which ultimately may result in serious consequences for population and community dynamics in aquatic ecosystems.

Conclusion

Using an experimental approach with semi-natural conditions, including either natural sunlight or natural sunlight with additional UVB, I showed that exposure to enhanced but ecologically relevant ambient UVB for different periods affects multiple fitness-relevant processes. Considerable negative effects were found on growth, immunity and sexually selected post-mating traits. Furthermore, I found a UVB-induced differential investment in pre- and post-mating traits directed towards pre-mating traits. Next to physiological consequences, I also revealed UVB-caused altered behavior regarding predator-prey interactions. UVB-stressed sticklebacks showed more risky behavior, i.e. promoted predator inspection, compared to their full-sibs exposed to natural sunlight.

Interestingly, no UVB effects on the expression of the breeding coloration in stickleback males were observed. By exposing full-sib brothers to either enhanced ambient UVB or solar radiation during growth, from early life on, I revealed a trade-off between pre-mating sexual ornamentation (red intensity of the breeding coloration) and a post-mating sperm trait (sperm number) in UVB-stressed individuals resulting in maintenance of the sexual attractiveness at the expense of sperm quantity.

Summary

Changing photic conditions are, next to global warming, the major aspect in terms of ongoing global climate change and mainly occur within the short-wave and high-energetic ultraviolet-B (UVB) part of the electromagnetic spectrum. Studies investigating the effect of UVB on organisms mostly use short-term exposure, i.e. single doses, and/or conducted experiments under laboratory settings, inevitably accompanied by artificial photic conditions. Studies using ecologically relevant levels of UVB in combination with standardized settings, e.g. genetic background of experimental animals or food availability, under outdoor conditions are scarce. In my thesis, I used a semi-natural outdoor setup to create two photic conditions, including either natural sunlight in combination with an additional UVB source or exclusively natural sunlight to examine the consequences of enhanced ambient UVB on the biology of three-spined sticklebacks (*Gasterosteus aculeatus*). I used different time spans of UVB exposure, applied at various life stages, to study short- and long-term phenotypic plastic responses of a teleost fish species confronted with a stressful environment. Three-spined sticklebacks inhabit shallow lentic and flowing aquatic habitats and are naturally exposed to ambient ultraviolet radiation. Whereas ultraviolet-A (UVA) radiation is used during visual communication, sticklebacks are incapable of perceiving UVB which makes them prone to disproportional increases in UVB.

Male sticklebacks develop a conspicuous red breeding coloration and face a high risk of sperm competition due to alternative reproductive strategies, i.e. stealing of fertilizations. Thus, in addition to studying effects on growth and immunity, male sticklebacks are suitable for studying resource allocation to pre- and post-mating sexually selected traits, which both determine an individual's reproductive success.

In my experiments, I showed that about two months as well as about seven and nine months of exposure to enhanced ambient UVB seriously impair growth. UVB affects various physiological processes, ranging from damage to DNA, enzymes, membrane proteins and lipids as well as increased photo-oxidative stress (formation of reactive oxygen species). Consequently, stressed individuals likely face higher energetic needs for costly repair mechanisms which may retard other physiological processes, e.g. digestion or growth.

I showed that exposure to enhanced ambient UVB for about two months has immunomodulatory effects in non-reproductive sticklebacks of both sexes. Stressed individuals had a lower relative spleen weight, i.e. splenosomatic index (SSI) together with a higher granulocyte-to-lymphocyte ratio of head-kidney leukocytes. The spleen and head-kidney represent major immune organs in teleost fishes. A lower SSI can be interpreted as a

downregulated adaptive immunity and a higher proportion of granulocytes indicates an upregulated innate immune response. Chronic exposure to enhanced UVB radiation may have promoted inflammatory processes (e.g. erythema), potentially accompanied by an intensification of the innate immune system. Consequently, in terms of the assumed trade-off between innate and adaptive immunity, the adaptive immunity may be reduced. However, whether the adaptive immune response ultimately is weakened by UVB needs further research.

About two months of exposure to enhanced ambient UVB caused considerable negative effects on post-mating sperm-related traits, i.e. smaller testes, lower sperm number and shorter sperm in reproductively active male sticklebacks. In contrast, UVB exposure for about nine months, from early-life during the major growth phase, had no significant effects on the measured post-mating traits. Though, after nine months of enhanced ambient UVB, I revealed a trade-off between the investment in a pre- and a post-mating trait in males raised under enhanced levels of UVB. More precisely, the intensity of the breeding coloration in UVB-stressed males was negatively correlated with sperm numbers, whereas this relationship was reversed for the control. In contrast to sticklebacks raised under natural sunlight, individuals exposed to stressful conditions were not able to uphold the honesty of a pre-mating sexually selected ornament. Thus, stressed individuals seem to invest resources in a pre-mating trait (breeding coloration) at the expense of a post-mating trait (sperm number). The absent differences between both exposure groups regarding the measured color traits suggest that stressed individuals maintain their sexual attractiveness in order to enhance the access to potential matings. By revealing this trade-off, I could provide the first experimental evidence for a differential allocation to costly pre- and post-mating traits as a result of exposure to a key environmental stressor in terms of climate change.

In summary, I showed the serious effects of enhanced but ecologically relevant levels of ambient UVB, representing an abiotic environmental stressor related to climate change, on multiple fitness-relevant traits in three-spined sticklebacks. I showed phenotypic plastic responses to enhanced UVB on physiological processes, i.e. growth, immunity, sperm-related traits as well as resource allocation to pre- and post-mating traits. Furthermore, I observed consequences of enhanced UVB on the behavior of sticklebacks regarding predator-prey interactions. In my thesis, I demonstrated that chronic exposure to enhanced ambient UVB affects fitness-relevant traits and may influence sexual selection processes under natural conditions. Moreover, enhanced ambient UVB has the potential to affect population and community dynamics in aquatic ecosystems.

Zusammenfassung

Veränderungen der photischen Verhältnisse sind, neben der globalen Erwärmung, der Hauptaspekt im Kontext des fortschreitenden Klimawandels und betreffen maßgeblich den kurzwelligen und hochenergetischen Ultraviolett-B (UVB) Bereich des elektromagnetischen Spektrums. Studien, die die Wirkung von UVB auf Organismen untersuchten, verwendeten meistens kurzzeitige Expositionen, d. h. Einzeldosen, und/oder führten Experimente unter Laborbedingungen und artifizieller Beleuchtung durch. Untersuchungen, die ökologisch relevante UVB-Strahlung in Kombination mit standardisierten Experimentalbedingungen, z. B. bekannter genetischer Hintergrund von Versuchstieren oder standardisierte Fütterung, bei gleichzeitiger Nutzung natürlicher Freilandbedingungen beinhalten, sind äußerst selten.

In meiner Doktorarbeit habe ich einen Experimentalaufbau unter semi-natürlichen Bedingungen im Freiland verwendet, um zwei unterschiedliche photische Bedingungen zu generieren. Ein Ansatz beinhaltete natürliches Sonnenlicht in Kombination mit einer zusätzlichen UVB-Quelle, wohingegen in einem zweiten Ansatz ausschließlich natürliches Sonnenlicht verwendet wurde. Mithilfe dieser zwei Bedingungen habe ich die Auswirkungen erhöhter, aber ökologisch relevanter UVB-Strahlung, auf unterschiedliche Merkmale des Dreistachligen Stichlings (*Gasterosteus aculeatus*) untersucht. Dazu habe ich Versuchstiere in verschiedenen Lebensphasen für unterschiedliche Zeitspannen UVB-Strahlung ausgesetzt, um kurz- und langfristige phänotypisch plastische Reaktionen zu erforschen.

Dreistachlige Stichlinge bewohnen flache, stehende und fließende aquatische Lebensräume und sind somit ultravioletter Umgebungsstrahlung ausgesetzt. Sie können Ultraviolett-A (UVA)-Strahlung wahrnehmen und nutzen diese während der visuellen Kommunikation. UVB hingegen kann von Dreistachligen Stichlingen nicht visuell wahrgenommen werden, wodurch sie anfällig gegenüber einem überproportionalen Anstieg von UVB-Strahlung im Umgebungslicht sind.

Männliche Stichlinge entwickeln während der reproduktiven Phase eine auffällige rote Brutfärbung und sind aufgrund alternativer Fortpflanzungsstrategien, wie dem Erschleichen von Befruchtungen, einem hohen Spermienkonkurrenzrisiko ausgesetzt. Zusätzlich zur Erforschung von Effekten auf grundlegende physiologische und immunologische Prozesse, z. B. Wachstum und Immunantworten, eignen sich männliche Stichlinge daher auch zur Untersuchung der Investition von Ressourcen in sexuell selektierte Merkmale, mit Relevanz vor und nach der Verpaarung, die den Fortpflanzungserfolg eines Individuums bestimmen. In meinen Experimenten konnte ich zeigen, dass sowohl etwa zwei Monate als auch sieben und

neun Monate Exposition gegenüber erhöhtem UVB das Wachstum stark beeinträchtigen. Verschiedene physiologische Prozesse werden von UVB beeinflusst, was zu Schäden an DNA, Enzymen, Membranproteinen und Lipiden, bis hin zu erhöhtem photooxidativem Stress (Bildung reaktiver Sauerstoffradikale) führen kann. Folglich haben gestresste Individuen wahrscheinlich einen höheren energetischen Bedarf durch kostenintensive Reparaturmechanismen, die wiederum andere physiologische Prozesse, z. B. Verdauung oder Wachstum, beeinträchtigen können.

Ich konnte zeigen, dass eine ca. zweimonatige Exposition gegenüber erhöhtem UVB immunmodulatorische Wirkungen bei nicht-reproduktiven Stichlingen beider Geschlechter hat. Gestresste Individuen hatten ein niedrigeres relatives Milzgewicht (splenosomatischer Index, SSI), zusammen mit einem höheren Granulozyten-Lymphozyten-Verhältnis von Kopfnieren-Leukozyten. Milz und Kopf-Niere sind wichtige Immunorgane bei Fischen. Ein niedrigerer SSI könnte als herunterregulierte adaptive Immunität interpretiert werden und ein höherer Anteil an Granulozyten zeigt eine hochregulierte Antwort des angeborenen Immunsystems an. Chronische Exposition gegenüber verstärkter UVB-Strahlung könnte entzündliche Prozesse (z. B. Erytheme) auslösen, die möglicherweise mit einer Intensivierung des angeborenen Immunsystems einhergehen. Folglich scheint im Hinblick auf einen angenommenen *trade-off* zwischen angeborenem und adaptivem Immunsystem die Antwort des adaptiven zugunsten des angeborenen Immunsystems verringert zu werden. Ob diese Effekte auf das Zusammenspiel von angeborenen und adaptiven Immunantworten jedoch bedeuten, dass die adaptive Immunantwort letztendlich durch UVB geschwächt wird, bedarf weiterer Forschung.

Bei reproduktiv aktiven männlichen Stichlingen führten ungefähr zwei Monate Exposition gegenüber erhöhtem UVB zu erheblichen negativen Auswirkungen auf spermienbezogene Merkmale in Form von kleineren Hoden, geringerer Spermienanzahl und kürzeren Spermien. Im Gegensatz dazu hatte die UVB-Exposition für etwa neun Monate, während der Hauptwachstumsphase, keine signifikanten Auswirkungen auf die gemessenen spermienbezogenen Merkmale. Jedoch konnte ich nach neun Monaten erhöhter UVB-Bestrahlung bei männlichen Stichlingen einen *trade-off* zwischen der Investition in ein Merkmal mit Funktion vor und ein anderes mit Funktion nach der Verpaarung zeigen. Genauer gesagt korrelierte die Intensität der Brutfärbung bei UVB-gestressten Männchen negativ mit der Spermienanzahl, während dieser Zusammenhang für die Kontrollgruppe umgekehrt war. Im Gegensatz zu Stichlingen, die unter natürlichem Sonnenlicht aufgezogen wurden, waren Individuen, die stressreichen Bedingungen ausgesetzt waren, nicht in der Lage die Ehrlichkeit eines sexuell selektierten Ornaments aufrechtzuerhalten. Gestresste Individuen scheinen

Ressourcen in ein Merkmal mit Relevanz vor der Paarung (Färbung) auf Kosten eines Merkmals mit Funktion nach der Paarung (Spermienzahl) zu investieren. Die fehlenden Unterschiede zwischen beiden Expositionsgruppen in Bezug auf die gemessenen Farbmerkmale legen nahe, dass gestresste Individuen ihre sexuelle Attraktivität aufrechterhalten, um Zugang zu potenziellen Paarungen zu erhalten. Durch das Aufdecken eines *trade-offs* konnte ich den ersten experimentellen Nachweis für unterschiedliche Investitionen in kostspielige Merkmale mit Bedeutung vor und nach der Paarung liefern, verursacht durch die Exposition gegenüber einem elementaren Umweltstressor.

Zusammenfassend konnte ich die schwerwiegenden Auswirkungen erhöhter, aber ökologisch relevanter UVB-Strahlung, welche einen abiotischen Umweltstressor im Zusammenhang mit dem Klimawandel darstellt, auf mehrere fitnessrelevante Merkmale bei Dreistachligen Stichlingen zeigen. Ich habe phänotypisch plastische Reaktionen auf erhöhte UVB-Bestrahlung hinsichtlich physiologischer Prozesse, d. h. Wachstum, Immunität und spermienbezogene Merkmale, sowie auf die Investition von Ressourcen in Merkmale mit Relevanz vor- und nach der Paarung gezeigt. Darüber hinaus habe ich Auswirkungen von verstärktem UVB auf das Verhalten von Stichlingen in Bezug auf Interaktionen zwischen Räubern und Beutetieren zeigen können. In meiner Doktorarbeit konnte ich belegen, dass sich die chronische Exposition gegenüber erhöhtem UVB in der Umgebung auf die Ausprägung fitnessrelevanter Merkmale auswirkt und sexuelle Selektionsprozesse unter natürlichen Bedingungen beeinflussen kann. Abschließend ist davon auszugehen, dass sich erhöhte UVB-Strahlungsbedingungen entscheidend auf Populations- und Lebensgemeinschaftsdynamiken in aquatischen Ökosystemen auswirken können.

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