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„Are anthropogenic stressors breaking the symmetries of the pelvic complex in the threespine stickleback *Gasterosteus aculeatus* Linneaus, 1758? "

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Abstract

The threespine stickleback (*Gasterosteus aculeatus*) is a teleost fish with a high phenotypic and ecological plasticity. It inhabits marine, brackish and freshwater habitats. These fish posses bony lateral plates instead of scales. These lateral plates along with the two dorsal spines, the pelvic girdle and the two ventral/pelvic spines form a defensive complex protecting against gape-limited predators. The paired elements of the defensive complex are very symmetric in populations under a high predatory pressure and reduced and more asymmetric under a low predatory pressure. This study explores the effect of anthropogenic stressors (decreased pH and/or increased temperature as expected at the end of the century), habitat (stream-lake) and natural selection (predatory pressure) onto the symmetry of pelvic structures in the threespine stickleback. For this purpose oceanic and freshwater populations were investigated. Scans of both sides of the pelvic girdle were taken and measured with Mathematica after the set of landmarks. All populations generally showed low differences in the symmetry in all investigated pelvic traits indicating that these structures are canalized. However there were differences along a predatory gradient. The populations under low to very low pressure were more asymmetric than the population under a high pressure in most investigated traits. Differences found between population inhabiting lotic and lentic habitats may also be due to differences in predatory pressure. Specimens reared under elevated CO2 did not significantly differ from the CO2 control group. The fish raised under elevated temperature (21 °C) differed significantly from the group kept under ambient temperature (17 °C) in the width of the ascending process and in pelvic spine length. Although the other traits of the pelvic girdle were not significantly asymmetric, the asymmetries in these two traits indicate that elevated temperature may decrease the efficiency of the defensive complex of the threespine sticklebacks.

Keywords: *Gasterosteus aculeatus*, phenotypic plasticity, defensive complex, pelvic girdle, anthropogenic stressors, natural selection

Zusammenfassung

Der dreistachelige Stichling (*Gasterosteus aculeatus*) ist ein Teleost mit einer hohen phänotypischen und ökologischen Plastizität. Er bewohnt marine Habitate, Brachwasser- und Süßwasserhabitate. Diese Fische besitzen Knochenplatten anstatt von Schuppen. Diese Lateralplatten formen mit den zwei Dorsalstacheln, dem Beckengürtel und den zwei Ventral-/Pelvisstacheln den Defensivkomplex, der gegen Prädatoren, die durch ihre Maulgröße limitiert sind, verteidigt. Die paarigen Elemente des Defensivkomplexes sind sehr symmetrisch in Populationen unter hohem Raubdruck, und reduziert und asymmetrischer unter niedrigem Raubdruck. Diese Studie untersucht den Effekt von anthropogenen Stressoren (verminderter pH und/oder erhöhte Temperatur wie sie am Ende des Jahrhunderts erwartet werden), Habitat (Fluss-See) und natürlicher Selektion (Raubdruck) auf die Symmetrie der Beckengürtelstrukturen des dreistacheligen Stichlings. Zu diesem Zweck wurden ozeanische Populationen und Süßwasserpopulationen untersucht. Scans der beiden Seiten des Beckengürtels wurden gemacht und mit Mathematica gemessen, nachdem Landmarks gesetzt wurden. Generell zeigten alle Populationen geringe Unterschiede in der Symmetrie in allen untersuchten Merkmalen. Das zeigt, dass diese Strukturen kanalisiert sind. Es gab Unterschiede entlang eines Predatorengradienten. Die Populationen unter niedrigem bis sehr niedrigem Raubdruck waren in den meisten untersuchten Merkmalen asymmetrischer als die Population unter hohem Raubdruck. Die gefundenen Unterschiede zwischen den Populationen, die lotische und lentische Habitate bewohnen, sind vielleicht auch durch Unterschiede im Raubdruck bedingt. Individuen, die unter erhöhtem CO2 aufgezogen wurden, unterschieden sich nicht signifikant von der CO2 control Gruppe. Die Fische, aufgezogen bei erhöhter Temperatur (21 °C), unterschieden sich signifikant in der Breite des aufsteigenden Processus und in der Länge der Ventralstacheln von der Gruppe, die bei Umgebungstemperatur (17 °C) aufgezogen wurde. Obwohl die anderen Merkmale des Beckengürtels nicht signifikant asymmetrisch waren, zeigt die Asymmetrie dieser beiden Merkmale, dass erhöhte Temperaturen vielleicht die Effizienz des Defensivkomplexes des dreistacheligen Stichlings vermindern.

Schlüsselwörter: *Gasterosteus aculeatus*, Phänotypische Plastizität, Defensivkomplex, Beckengürtel, Anthropogene Stressoren, Natürliche Selektion

1. Introduction

The threespine stickleback is a teleost fish with a high phenotypic and ecological plasticity. It inhabits freshwater, marine and brackish water habitats all around the Northern hemisphere (Münzing, 1963; Bell and Foster, 1994; Wootton, 2009) . Nevertheless its main distribution can be found in the ocean (Münzing, 1963; Wootton, 2009). Freshwater populations stem from numerous invasion events of oceanic (anadromous and marine) threespine sticklebacks (Münzing, 1963; Bell and Foster, 1994; Myhre and Klepaker, 2009; Wootton, 2009; Schluter et al., 2010; Wark and Peichel, 2010; Aguirre and Bell, 2012; Klepaker et al., 2012; Terekhanova et al., 2014). The marine origin of the threespine stickleback can be supported through genetic studies that detected that freshwater populations are genetically less diverse than oceanic populations (Mäkinen et al., 2006; Cresko et al., 2007; DeFaveri et al., 2011, 2013; Terekhanova et al., 2014). The invasion of freshwater habitats started 12 000-10 000 years ago at the end of the last Peleistocene glaciation when inland waters could be re-colonized during warmer periods from refugial areas (Mäkinen et al., 2006; Wootton, 2009; Schluter et al., 2010; Leinonen et al., 2012; Terekhanova et al., 2014). The threespine stickleback was introduced several times in European water bodies (summarized in Ahnelt 1983; Ahnelt et al. 1995; Paepke 2002). In Austria first introductions are documented from the 19th century (about 1860) (Ahnelt et al., 1995, 1998; Ahnelt et al., 2006 a; Ahnelt et al., 2006 b). These allochtonous populations established in Lake Constance and along the Rhine and Danube river systems (Ahnelt et al., 1998).
Sticklebacks can be crossed and reared in the laboratory and have a short generation time, which makes them a great model organism for many different fields (Cresko et al., 2007).

1.1. Ecotypes

There are three different ecotypes of threespine sticklebacks. The marine populations spend their whole life in the ocean, anadromous populations over-winter in the sea and breed in freshwaters (Bell and Foster, 1994; Aguirre and Bell, 2012) and the freshwater populations are inhabiting freshwater habitats (Paepke, 2002; Wootton, 2009; Spence et al., 2012; Terekhanova et al., 2014). Body shape can extremely differ between anadromous and freshwater, stream and lake or benthic and limnetic populations (Cresko et al., 2007; McGuigan et al., 2010; Rogers et al., 2012) reflecting the different habitat uses (Aguirre and Bell, 2012). Differences in body shape can be caused by abiotic as well as biotic characters of a habitat (McGuigan et al., 2010) and they can sometimes be associated with an alteration in behaviour (Spoljaric and Reimchen, 2007) and in sensory systems (Myhre and Klepaker, 2009; Wark and Peichel, 2010).

1.2. Plate morphs

There are three different morphs discernible based on the plate number: the completely, partially and low plated morph (Münzing, 1963; Bell and Foster, 1994; Bergstrom and Reimchen, 2000; Wootton, 2009; Grøtan et al., 2012; Leinonen et al., 2012; Spence et al., 2012).

The marine completely plated morph that is considered as the ancestral plate morph (Münzing, 1963; Ahnelt et al., 1998; Reimchen, 2000; Spoljaric and Reimchen, 2007; Grøtan et al., 2012; Shikano et al., 2013) is the most abundant morph and has a complete row of lateral plates from the head to the base of the caudal fin (Reimchen, 2000; Wootton, 2009; Leinonen et al., 2012). On the caudal peduncle these plates from a keel (Reimchen, 1983, 2000; Paepke, 2002; Wootton, 2009; Klepaker et al., 2012), long and robust spines (Godin and Valdron Clark, 1997; Cresko et al., 2004) and a complete pelvic girdle (Shapiro et al., 2004; Terekhanova et al., 2014). This morph usually shows a row of 35 lateral plates (Reimchen, 1983). It is generally associated with a high salinity and low temperatures (Münzing, 1963; Reimchen, 2000; Cresko et al., 2007; Wootton, 2009). The anadromous and freshwater stickleback populations were derived from marine specimens (Münzing, 1963; Bell and Foster, 1994; Ahnelt et al., 1998; Wootton, 2009; McGuigan et al., 2010; Aguirre and Bell, 2012). The freshwater populations often show reduced body armour, which can lead to shorter dorsal and smaller pelvic spines, resulting in a poorer defence against predators (Klepaker et al., 2012; Shikano et al., 2013). One of the reasons for a reduction in bony elements may be the calcium concentration that is much lower in freshwater than in marine habitats (Reimchen, 1983; Ahnelt et al., 1998; Bell et al., 2007; Coyle et al., 2007; Myhre and Klepaker, 2009; Wootton, 2009; Schluter et al., 2010; Nosil and Schluter, 2011; Spence et al., 2012; Terekhanova et al., 2014).

The plate morph most associated with freshwater habitats is the low plated one (Münzing, 1963; Bergstrom and Reimchen, 2000; Myhre and Klepaker, 2009; Wootton, 2009; Grøtan et al., 2012; Terekhanova et al., 2014). The bony armour of the low plated morph usually consist of less than nine lateral plates per side and usually lacks a caudal keel (Reimchen, 1983; Bergstrom and Reimchen, 2000; Wootton, 2009) although low plated specimens that possessed one were also found (Paepke, 2002; Ahnelt et al., 2006 b). The poor availability of calcium that is necessary for the development of the arming in freshwaters may be the reason for the reduction of bony elements (Myhre and Klepaker, 2009; Wootton, 2009), but see also Klepaker et al (2012).

The partially plated specimens are intermediate and display an incomplete row of lateral plates along their body including caudal plates (Münzing, 1963; Reimchen, 1983; Bell and Foster, 1994; Wootton, 2009; Spence et al., 2012). This reduction in lateral plates may cohere with an overall reduction in armour (Aguirre and Bell, 2012).

Skeletal morphology is under a genetic control, which differs between trait classes and the specific control of individual elements of the trait classes (Miller et al., 2014). The lateral plate morph is associated with a genetic locus, *Eda* that is coding for ectodysplasin, a signal protein that is involved in the development of teeth and dermal bones of mammals (Peichel, 2005; Wootton, 2009). Low morphs demonstrate a different allele of *Eda* compared to the complete morph (Cresko et al., 2004, 2007; Peichel, 2005; Myhre and Klepaker, 2009; Wootton, 2009; Schluter et al., 2010).

1.3. Defensive Complex

The phenotypic diversity can be best studied on their predatory defense structures. Threespine sticklebacks posses bony plates, each lying on top of a myomere, arranged along their body sides, two large dorsal spines and ventrally a pair of pelvic spines (Bell and Foster, 1994; Bergstrom and Reimchen, 2000, 2003; Paepke, 2002; Coyle et al., 2007). The defensive complex in detail generally consists of 20 elements that are divided into three units: The first unit includes the first and the second dorsal spine along with their pterygiophores, the second unit comprises five lateral plates (LP) LP4-LP8 and the third unit contains the pelvic girdle with its three processes and two pelvic spines (Ahnelt et al., 2006 b). The total number of lateral plates may be reached at a standard length of about 30 mm (Schluter et al., 2010).

The lateral plates are under selective pressure, because they protect the body against puncture during predatory handling (Bergstrom and Reimchen, 2000; Reimchen and Bergstrom, 2009) and are able to interfere with pharyngeal actions of gape-limited predators (Reimchen, 1994, 2000; Bergstrom and Reimchen, 2003; Schluter et al., 2010; Lescak et al., 2011). These plates can be divided into structural (overlie myomere four to eigth) and nonstructural plates (Bergstrom and Reimchen, 2003; Ahnelt et al., 2006 a). The structural plates are located next to the dorsal and pelvic spines and support the resistance against the deflection of the spines (Reimchen, 1994, 2000, Bergstrom and Reimchen, 2000, 2003). Asymmetries are in fact more common in nonstructural plates compared to the structural plates (Allenbach, 2011; Loehr et al., 2013), which could be an indication for a strong selective pressure on the symmetry of structurally important plates (Bergstrom and Reimchen, 2000, 2003). The dorsal and pelvic spines are connected to bony pterygiophores (Paepke, 2002; Bergstrom and Reimchen, 2003). The dorsal as well as the pelvic spines can be brought into an erect position to decrease the risk of being eaten by a gape-limited predator (Bell and Foster, 1994; Peichel et al., 2001; Reimchen and Nosil, 2002; Peichel, 2005; Klepaker et al., 2012; Miller et al., 2015). Through an increased cross-sectional diameter the swallowing success of predators is reduced (Reimchen, 1983, 2000; Sillett and Foster, 2000; Shapiro et al., 2004). The importance of the spines can be demonstrated by the fact that these structures are elongated under high predatory pressure and reduced in habitats without predators (Reimchen, 1983; Bell and Foster, 1994; Reimchen and Nosil, 2002). Some piscivorous predators are specifically preying on sticklebacks with reductions in their defensive complex (Lescak et al., 2011). On the other hand invertebrate predators like dragonfly nymphs that catch their prey by grasping their appendages can lead to reduced armour to minimize the attack surface (Reimchen, 1994; Ziuganov and Zotin, 1995; Reimchen and Nosil, 2002; Nosil and Schluter, 2011; Lescak et al., 2012).

The spines are structurally supported by the overlap between the structural plates and the dorsal basal plates and the ventral ascending process (Bergstrom and Reimchen, 2002, 2003; Klepaker et al., 2012). In addition this overlap enables a dispersion of forces during predator manipulation (Bergstrom and Reimchen, 2002). During the process of lateral plate reduction the nonstructural plates are the first to be reduced and the structural plates are the last to be lost (Reimchen, 1994; Bergstrom and Reimchen, 2003; Ahnelt et al., 2006 a). The symmetry in lateral plates is associated with a great fish preda-

tion (Bergstrom and Reimchen, 2000; Reimchen and Nosil, 2001a; Nosil and Reimchen, 2005; Reimchen and Bergstrom, 2009). But some studies suggest that asymmetry might be increased under a high avian predation (Reimchen, 1983). However asymmetric specimens may also exhibit different behaviour than symmetric ones (Reimchen and Nosil, 2001a; Reimchen and Bergstrom, 2009).

1.4. Pelvic Girdle

The ancestral pelvic girdle comprises four paired elements: ascending, anterior and posterior processes and the pelvic spine (Bell and Harris, 1985; Bell and Foster, 1994; Brown, 1994; Bell et al., 2007; Lescak et al., 2011, 2012). The ascending process can be forked and usually shows a range of one to three forks (Reimchen, 1983; Bell and Foster, 1994). The pelvis supports the pelvic spines and protects internal organs (Klepaker et al., 2012). This complex is developed highly symmetric and robust under predatory pressure and its development may not be terminated at a standard length under 20 mm (Bell et al., 2007). The pelvic girdle can be reduced in size and complexity and can differ between the different stickleback ecotypes (Nelson, 1971; Ziuganov and Zotin, 1995; Paepke, 2002; Cresko et al., 2004; Lescak et al., 2011; Aguirre and Bell, 2012; Klepaker et al., 2012). The sometimes very extensive reduction is a widespread phenomenon and it has evolved repeatedly in the threespine stickleback (Blouw and Boyd, 1992; Shapiro et al., 2004, 2006; Peichel, 2005; Bell et al., 2007; Chan et al., 2010; Lescak et al., 2012; Rogers et al., 2012). Specimens with a reduced pelvis have already been found in the Miocene stickleback, *Gasterosteus doryssus* (Blouw and Boyd, 1992; Shapiro et al., 2004; Bell et al., 2007).

Vestigial pelvic phenotypes as well as general arming reduction can usually be found in freshwater habitats without predatory fish and a low ionic concentration (Blouw and Boyd, 1992; Bell and Foster, 1994; Bell et al., 2007; Coyle et al., 2007; Myhre and Klepaker, 2009; Klepaker et al., 2012, 2013). Specimens with a complete pelvis may even have different ecological roles than individuals with a reduced pelvis (Reimchen, 1997; Klepaker et al., 2012) and the same applies for asymmetric and symmetric specimens (Reimchen and Nosil, 2001a, 2001b; Reimchen and Bergstrom, 2009).

For example a behavioural difference observed was that fish with asymmetric phenotypes are more benthic, which could be a mechanism to avoid exposure of specific parasites with pelagic primary host (Reimchen and Nosil, 2001a, 2001b). Besides their role as a defense against predators pelvic spines are used against territorial rivals and intruders (Blouw and Boyd, 1992). Pelvic spine symmetry in males is important, because it may even play a role in sexual selection (Reimchen, 1997; Mazzi and Bakker, 2003; Mazzi et al., 2004). These spines are put into an erect position during courtship to attract females and more symmetric males have a higher reproductive success (Blouw and Boyd, 1992; Paepke, 2002; Mazzi and Bakker, 2003; Mazzi et al., 2004; Bakker et al., 2006).

1.5. Stressors

Stressors can be divided into three groups: physical (e.g. temperature, water staining), biological (infection, predatory pressure) and chemical (acidification, pesticides) (Allenbach, 2011).

1.5.1. Temperature

Temperatures are an abiotic factor that has a high control over fish activity, metabolic rate and oxygen content (Beitinger and Fitzpatrick, 1979). Environmental temperature can have a great impact on morphology of fish (Valentine et al., 1973). It can have a direct impact through an increased metabolism (Dittmar et al., 2014) or an indirect impact by changing physiochemical characteristics of the water (Moran et al., 2010; Ramler et al., 2014). Further higher temperatures can have an influence on maturation and reproduction, because of an increased growth rate (Sokołowska and Kulczykowska, 2009; Lee et al., 2012; Ramler et al., 2014; Pimentel et al., 2016). It can also alter host-parasite interactions leading to an increase of infectious diseases (Macnab and Barber, 2012; Dittmar et al., 2014; Schade et al., 2014 b). Ramler *et al*. (2014) found effects on the body shape of juvenile threespine sticklebacks raised under different temperature conditions.

1.5.2. Acidification

An increase of atmospheric CO2 resulting in an increased acidity is known to have an negative impact on the metabolism and the development of fish probably leading to deformities and pathological conditions (Jagoe and Haines, 1985; Pimentel et al., 2014 a; Pimentel et al., 2014 b; Schade et al., 2014 a; Ahnelt et al., 2016; Pimentel et al., 2016). This stressor is going to be of high relevance due to an increased amount of green house gas in the atmosphere through anthropogenic impact. Threespine sticklebacks show a high pH tolerance and are able to inhabit waters with a pH as low as 3.5 (Mazzi and Bakker, 2001; Taugbøl et al., 2014). Freshwater populations are confronted with lower pH levels than the marine ancestral sticklebacks (Schade et al., 2014 a). They can cope with a great bandwidth of temperatures (Moran et al., 2010; Dittmar et al., 2014; Schade et al., 2014 b; Metzger et al., 2016) and water conductivities (Wootton, 2009).

1.5.3. Predation

Predation is a very important factor for the development of morphological structures. It acts as a mechanism of natural selection, has a huge impact on the survival and divergence of species (Bergstrom and Reimchen, 2003), and can be the reason for high phenotypic diversity (Nosil and Reimchen, 2005; Miller et al., 2015). Predators can influence behaviour and morphology of their prey through natural selection (Godin and Valdron Clark, 1997). Klepaker at al. (2012) for example found reductions in the pelvic structures of Alaskan stickleback populations only in the absence of predators, indicating that predatory pressure had a higher impact on the development than the availability of important minerals for skeletal development.

1.6. Asymmetry

There are three types of asymmetry: directional asymmetry, fluctuating asymmetry and antisymmetry (Jagoe and Haines, 1985; Mazzi and Bakker, 2001; Graham et al., 2010; Allenbach, 2011; Loehr et al., 2013; Kenney and von Hippel, 2014).

Antisymmetry (AS) is the asymmetry of a trait towards a random side (Jagoe and Haines, 1985; Allenbach, 2011; Loehr et al., 2013; Kenney and von Hippel, 2014).
Directional asymmetry (DA), a developmental difference of a trait between the sides (Jagoe and Haines, 1985; Reimchen, 1997; Bergstrom and Reimchen, 2000; Reimchen and Nosil, 2001a; Allenbach, 2011; Kenney and von Hippel, 2014). This kind of asymmetry should not be used as a marker for environmental stress, because it has an assumed genetic component (Reimchen, 1997; Mazzi and Bakker, 2001; Reimchen and Nosil, 2001a) as well as antisymmetry (Allenbach, 2011). Nevertheless DA may also be an indicator for stress experienced during development in traits that show a low heritability and therefore may also reflect quality and condition of individuals (Mazzi and Bakker, 2001; Reimchen and Nosil, 2001a; Graham et al., 2010; Allenbach, 2011; Loehr et al., 2013). The same could be the case in antisymmetry (Graham et al., 2010; Allenbach, 2011).
Fluctuating asymmetry (FA), which is defined as small random deviations from perfect bilateral symmetry, can be used to detect developmental instability (Valentine et al., 1973; Jagoe and Haines, 1985; Reimchen, 1997; Bergstrom and Reimchen, 2000, 2002, 2003; Mazzi and Bakker, 2001; Mazzi et al., 2004; Reimchen and Bergstrom, 2009; Graham et al., 2010; Allenbach, 2011; Loehr et al., 2013; Kenney and von Hippel, 2014). It may also be used as an indicator for habitat quality (Valentine et al., 1973; Bergstrom and Reimchen, 2002, 2003). Nevertheless FA may be heritable although its degree of heritability is predicted to be low (Mazzi and Bakker, 2001; Bergstrom and Reimchen, 2002; Loehr et al., 2013).
Traits of functional importance (for example the components of the defensive complex in sticklebacks) should exhibit a decreased FA, because of selection by predatory pressure, even though specimens are under a high developmental stress (Bergstrom and Reimchen, 2003; Allenbach, 2011).

1.7. Aim

The aim of the study is to determine whether anthropogenic stressors like decreased pH and/or increased temperature increase asymmetries in pelvic structures in the threespine stickleback. The effect of anthropogenic stressors will be compared to the effects of natural selection (predatory pressure). In addition populations of lake and stream habitats were compared.

2. Material and Methods

Overall 428 specimens of threespine sticklebacks (*Gasterosteus aculeatus)* were examined. The material comprises from six European populations, four freshwater populations from Austria, one anadromous population from Denmark and one oceanic population from Germany (Fig.1; Fig 2; Fig 3; Table 1).

Austria: Aiglbach near Fischamend, Neubach (Himberg), Lustenauer Kanal near Lustenau and Fussacher Bay. All of the Austrian populations of freshwater sticklebacks were introduced) (Ahnelt et al., 1995, 1998; Ahnelt et al., 2006 a; Ahnelt et al., 2006 b).

Denmark: A natural migrating anadromous population from the Kisbaek creek (Bred Å system).

Germany: A natural population from the Sylt-Rømø Bight (North Sea). From these threespine stickleback population juveniles were reared under elevated temperature (Ramler et al., 2015) and decreased pH (Schade et al., 2014) respectively. Besides the adults also the juveniles were investigated.

These locations were chosen to detect the potential morphological effects of different predatory pressures in different waterbodies (lake, stream) and also to detect probable differences between introduced and natural stickleback populations. In addition the effects of low pH and high temperatures were examined through the offspring of the oceanic population. The number of individuals in a population ranged from 15 up to 115 (Table 1).

Table 1: Description of the six investigated populations of *Gasterosteus aculeatus*.

Population	AB: Aiglbach	HN: Neubach	LK: Lustenauer Kanal	FUB: Fussacher Bay	BKD: Brede Å system, Kisbaek creek	CO2 parents: marine adults from Sylt-Rømø Bight of CO2 group	CO2: offspring of marine adults from Sylt-Rømø Bight of CO2 group	T parents: marine adults from Sylt-Rømø Bight of temperature group	T: offspring of marine adults from Sylt-Rømø Bight of temperature group
Habitat	stream; freshwater	stream; freshwater	stream; freshwater	lake; freshwater	lake; marine	lake; marine	aquaria	lake; marine	aquaria
Coordinates of sampling sites (Latitude, Longitude)	48°03'N, 16°35'E	48°05'39"N, 16°26'27"E	47°27'24"N, 9°40'15"E	47° 29'41"N, 9° 39'32"E	55°04'25" N, 9°01'17"E	55°03`N, 8°44`E	-	54°52` to 55°10` N, 8°20` to 8°40` E	-
Sampling date/month/year	25.08.1994; 10.5.1995	03.06.1997	13.06.2014	05/2008	04/2011	04/2012	-	-	-
Sample number	32	34	31	33; 12 males; 13 females; 8 undetermined	34; 17 males; 17 females	19 females	115; 56 CO2 control; 59 CO2 high	15; ten females; five males	115; 56 T 17; 59 T 21
Predatory pressure	very low	low	high	high	high	high	non	high	non
Preservation	glycerol	glycerol	ethanol	ethanol	ethanol	ethanol	glycerol	ethanol	glycerol

2.1. Sampling sites

2.2. Aiglbach, Austria (AB): introduced freshwater population

The sticklebacks were collected from a tributary of the River Danube on the 25.8.1994 and on the 10.5.1995 by H. Belanyecz, H. Keckeis and H. Ahnelt (48°03'N, 16°35'E) (Fig. 1). They were sampled by electrofishing gear dip net (0.5 cm mesh width). After that the fish were killed by an overdose of MS -222, fixed in 6% formol and were transferred into 75 % ethanol. They were cleared, stained in Alicarin Red S and stored in glycerol (Ahnelt et al., 1998).

From this introduced stream population 32 specimens were investigated in this study.

This locality is characterised by a very low predatory pressure.

2.3. Neubach near Himberg (HN): introduced freshwater population

The specimens were sampled on the 3.6.1997 (48°05'39"N, 16°26'27"E) and were treated the same way as the fish from Aiglbach (Fig. 1). They were stored in glycerol.

This stream population from a tributary of Lake Danube consisted of 34 individuals.

The predatory pressure at this sampling site is low.

2.4. Lustenauer Kanal (LK): introduced freshwater population

The 31 fish were collected by A. Dünser and A. Lunardo on the 13.6.2014 (47°27'24"N, 9°40'15"E).

The predatory pressure at this sampling site is high.

The population was stored in ethanol (75%).

2.5. Fussacher Bay (FUB): introduced freshwater population

The 33 specimens were sampled in May 2008 by A. Lunardo from a tributary of Lake Constance (47° 29'41"N, 9° 39'32"E).

The predatory pressure at this sampling site is high. Common predators of this lake are pike (*Esox lucius*), lake trout (*Salmo trutta lacustris*), zander (*Sander lucioperca*), cormorant (*Phalacrocorax carbo*) and great crested grebe (*Podiceps cristatus*).

The population consisted of 12 males, 13 females and 8 individuals with undetermined sex. Sex was determined by examination of the gonads.

All specimens were stored in ethanol (75%).

2.6. Brede Å system, Kisbaek creek (BKD): anadromous population

This population consisting of 34 individuals, 17 females and 17 males, was collected in April 2011 in Kisbæk (55°04'25"N, 9°01'17"E). The tributary of the River Brede Å discharges into the Sylt-Rømø Bight. The creek was at the sampling area approximately 1.5 m wide and showed a depth of up to 0.6 m.

All sticklebacks from this population were stored in ethanol (75%).

Threespine sticklebacks from this location are under a high predatory pressure.

2.7. Sylt-Rømø Bight

2.7.1. CO2 Group: marine adult population (CO2 parents), offspring reared under different CO2 levels (CO2)

The adult marine sticklebacks were caught on a day cruise with the RV "Mya" in April 2012 in Sylt-Rømø Bight, North Sea in Germany (55°03`N, 8°44`E) for a former ocean acidification experimental setting in Schade et al. 2014. Schade et al. built two acclimation groups. One group was kept in seawater with an ambient CO2 level of 482 ± 15µatm whereas the other one was kept in seawater with elevated CO2 level of 1.013 ± 122 µatm. They generated fertilized egg clutches and divided them into groups of different ambient CO2 levels. The control group was kept at CO2 level of 435 ± 27 µatm (ambient) (CO2 control) and the high group was confronted with elevated CO2 levels of 1.167 ± 176 µatm (CO2 high).

The pelvic girdle of the adult male individuals was destroyed during former investigations. Therefore only the females were used in this study. The standard length of the 19 adult specimens was measured except of seven individuals that were beheaded. Also the juvenile fish showed a damaged pelvis and so they had to be handled differently for later analyzes (described in Scanning and Geometric morphometrics).

The offspring of the marine sticklebacks consisted of 115 individuals. The CO2 control group included 56 and the CO2 high group covered 59 specimens.

The predatory pressure of marine environments is generally high so adult fish were confronted with a high predatory abundance before they were captured. The juvenile fish were kept in aquaria without any predators.

2.7.2. Temperature Group: marine adult population (T parents), offspring reared under different temperatures (T)

Wild adult fish have been caught in the Sylt-Rømø Bight (54°52`- 55°10` N, 8°20`- 8°40` E). The offspring of these specimens were investigated in Ramler et al. (2014). In this former study adult fish were kept in aquaria at temperatures of 17 °C. This temperature represents the mean North Sea surface temperature during the summer. Stickleback families were generated through a paternal half-sibling mating design. Egg clutches were divided into three different groups. Each group was reared under a different temperature (13, 17 and 21 °C). Only the offspring with the ambient temperatures of 17 °C (T 17) and the elevated temperature 21 °C (T 21) was used in this study.

The population consisted of 15 adult individuals, five males and ten females and 115 juvenile sticklebacks. Juvenile T 17 fish included 56 and T 21 covered 59 specimens.

The adult fish are generally under a high predatory pressure in the sea. Their offspring was kept under experimental conditions without any predation risk.

2.8. Predatory pressure:

These populations, except the one from Sylt-Rømø Bight, were chosen due to different predatory pressure: from very low to moderate and the high predation. In general, at high predatory pressure the defensive complex is well developed and highly symmetric, at low predatory pressure it is reduced and asymmetric (Reimchen, 1983, 2000; Ahnelt et al., 1998; Bergstrom and Reimchen, 2003). The predatory pressure is known to be high in FUB, BKD, parents CO2, parents T and low in AB and HN populations. The main predators were pike (*Esox lucius*), lake trout (*Salmo trutta lacustris*), zander (*Sander lucioperca*), cormorant (*Phalacrocorax carbo*) and great crested grebe (*Podiceps cristatus*). Sticklebacks from marine habitats are confronted with a higher predatory pressure than the freshwater populations (Schluter et al., 2010).

Figure 1: Sampling sites in Austria. 01: AB. 02: HN. Scale: 10 km. Modified after mapmaker.education.nationalgeographic.com (7.10.2016).

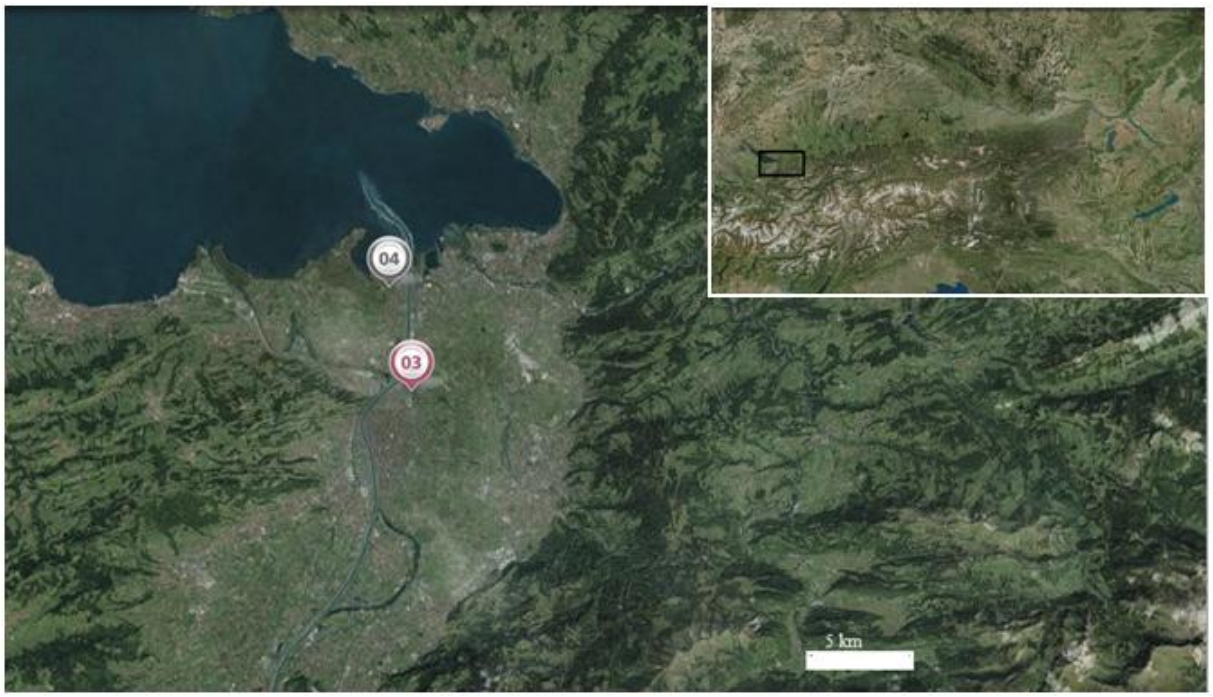

Figure 2: Sampling sites in Austria: 03: LK. 04: FUB. Scale: 5 km. Modified after map-maker.education.nationalgeographic.com (7.10.2016).

Figure 3: Sampling sites in Germany and Denmark. 05: BKD (Denmark). 06: CO2 parents (Germany). 06: T parents (Germany). Scale: 10 km. Modified after mapmaker.education.nationalgeographic.com (11.12.2016).

2.9. Data acquisition

2.9.1. Standard length measurements

The measurements of the standard length (SL) were taken with a digital calliper (Vogel Germany Messwerkzeugfabrik GmbH & Co. KG, Id.-No. C710120061). The SL was measured twice to the nearest of 0.01 mm. In case of a difference between these two values between 0.1 mm and 0.2 mm a third value was taken. If the difference between the measurements was higher than 0.2 mm, all values were discarded and at least two measurements were taken again. The mean of the measurements was used in the study.

Not all specimens of the adult anadromous threespine sticklebacks from the ocean acidification experiment could be measured, because they were beheaded during a former study.

2.9.2. Scanning

To show the pelvic girdle the part of the fish between head and tail was scanned using a flatbed scanner (Epson Perfection V330 Photo) (Fig. 4).

A plastic tube was sealed up at its base with plasticine. After that it was filled either with 100 % glycerol or 75 % ethanol depending on the preservation of the specimen. After the fish was placed in the tube for scanning it was covered by a white paper sheet to improve the contrast.

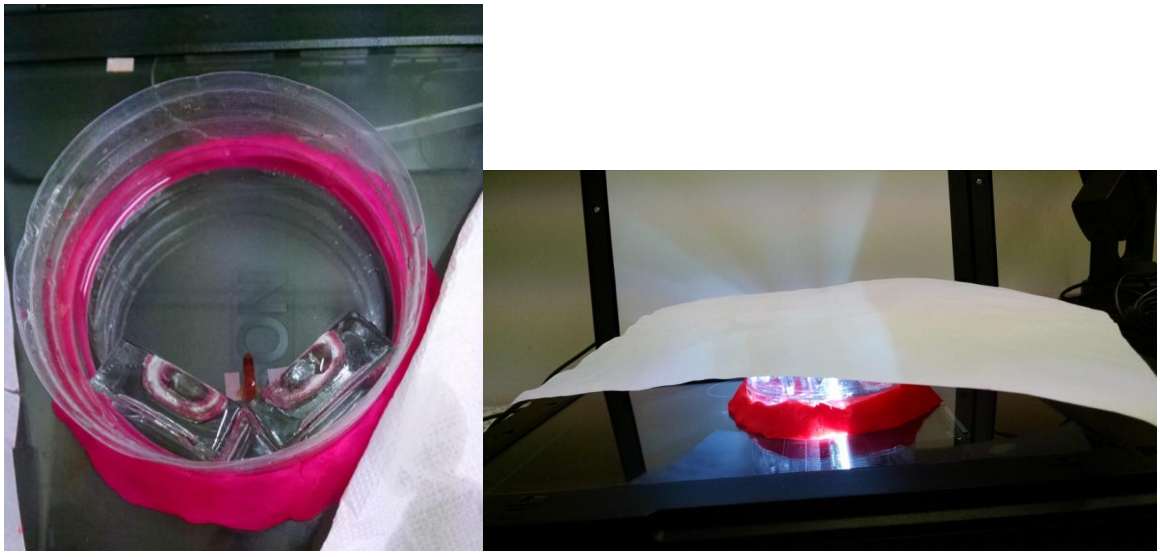

Figure 4: The set up of for the scanning process. The scanning process shown on a ventrally stabilised specimen (left). The tub was covered for a better contrast (right).

Every fish was scanned on its left, right and its ventral side except of the juveniles reared under increased and normal CO2 conditions. These specimens were not scanned on their ventral side, because of their cut pelvic girdle.

2.9.3. Geometric morphometrics

Geometric morphometrics is a statistical analysis, which can be used to visualize shapes and shape deformations (Mitteroecker and Gunz, 2009). In this study it was used for the measurement of pelvic traits. The scans were converted into tps-files by tps Util64 (James Rohlf). To measure the length of pelvic traits four landmarks (five in the juvenile fish from the CO2 setting) were set on the lateral sides of the fish and 15 landmarks were set on the ventral side of the specimens using tpsDig264 (James Rohlf; Table 2, Fig. 5).

Table 2: Description of landmarks 1-20.

Landmarks	
	Lateral
16	the anterior edge on the base of the pelvic spine
17	the most dorsal point of the ascending process
18	the most anterior point of the ascending process
19	the most posterior point of the ascending process
20	the tip of the pelvic spine; only set in juveniles from the CO2 group
	Ventral
1	the tip of the right pelvic spine
2	the tip of the left pelvic spine
3	the end of the right posterior process
4	the end of the left posterior process
5	the point between the ascending process and the right pelvic spine
6	the point between the ascending process and the left pelvic spine
7	the most anterior point of the right pelvic spine
8	the most anterior point of the left pelvic spine
9	the widest point of the right part of the anterior process
10	the widest point of the left part of the anterior process
11	the point on the suture at the widest part of the anterior process
12	the most anterior point of the right anterior process
13	the most anterior point of the left anterior process
14	the origin of the serrated part of the anterior process on the right side
15	the origin of the serrated part of the anterior process on the left side

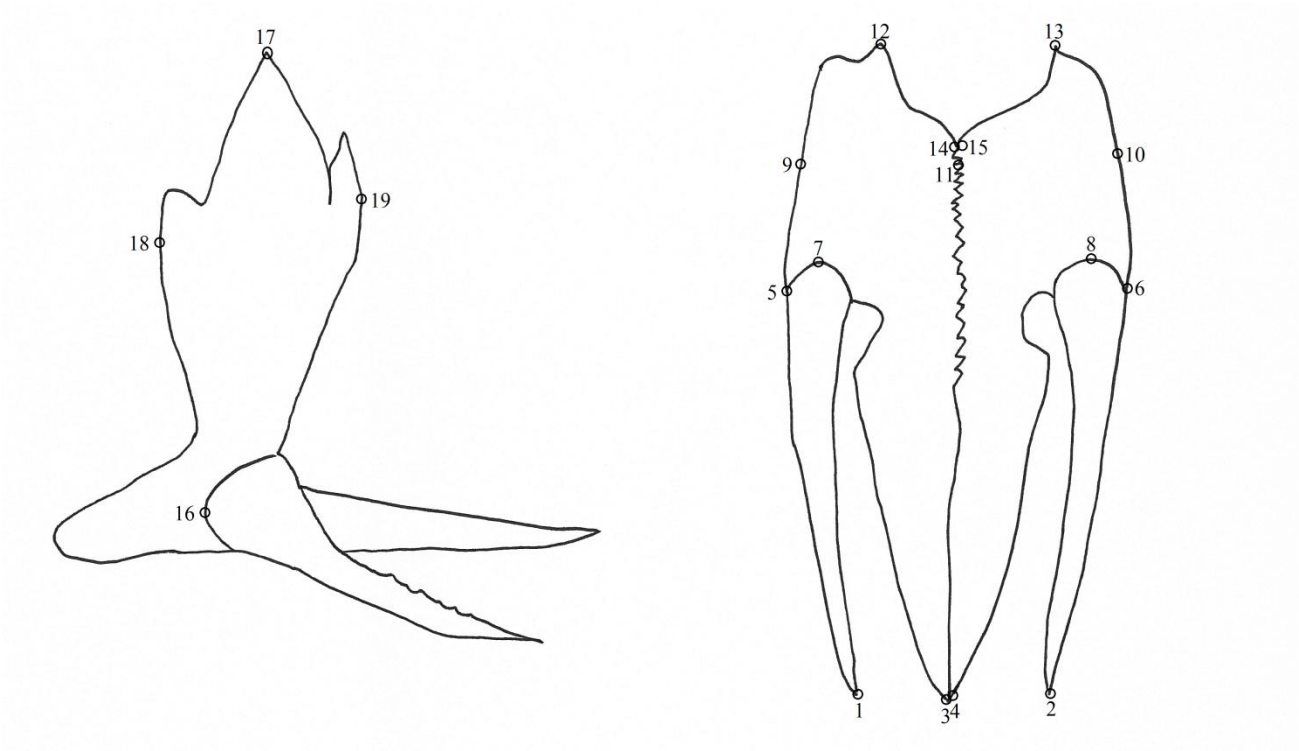

Figure 5: Scheme of the pelvic girdle of a threespine stickleback from a lateral (left) and ventral (right) view with the landmarks used in this study. 1 = tip of the right pelvic spine; 2 = tip of the left pelvic spine; 3 = end of the right posterior process; 4 = end of left posterior process; 5 = point between the ascending process and the right pelvic spine; 6 = point between the ascending process and the left pelvic spine; 7 = most anterior point of the right pelvic spine; 8 = most anterior point of the left pelvic spine; 9 = widest point of the right part of the anterior process; 10 = widest point of the left part of the anterior process; 11 = point on the suture at the widest part of the anterior process; 12 = most anterior point of the right anterior process; 13 = most anterior point of the left anterior process; 14 = origin of the serrated part of the anterior process on the right side; 15 = origin of the serrated part of the anterior process on the left side

2.9.4.Dorsal edge of the ascending processes

It was also investigated if the dorsal edge of the ascending process was forked (Fig. 6). These structures were investigated using a stereomicroscope (Nikon, SMZ800). Both sides of the fish were explored. Specimens with the same number of dorsal edges of the ascending branch on their left and right side were referred to as symmetric, whereas individuals with a deviating number of dorsal edges between the lateral sides were described as asymmetric.

Figure 6: Scheme of ascending process types depending on the number of dorsal edges. One dorsal edge (left), two dorsal edges (middle) and three dorsal edges (right).

2.9.5.Measurements and statistical analysis

Wolfram Mathematica 10.4 (Wolfram Research Inc., Champaign, IL, USA) was used to receive the pelvic traits. 14 traits were measured: ascending process length (ABL), ascending process width (ABW), pelvic spine length (PSL), posterior process length (PPL), anterior process length (APL), anterior process width (APW), length of posterior process to the midline of the anterior processes (PPLF), and length of the anterior process from the midline (FAP) (Table 3, Fig. 7). Each trait was measured on both sides of the fish to detect probable asymmetries. The measured distances consisted of two on the lateral sides (three in the juvenile specimens under different ambient CO2 levels) and of twelve on the ventral side. All of the juvenile sticklebacks reared under different CO2 pressure had a ventrally destroyed pelvic girdle and that is the reason why the pelvic spine length was measured from the lateral sides of the fish and all structures of the anterior and posterior process could not be estimated.

Because asymmetries between the lateral sides may be very small FA can be highly influenced by measurement errors (Bergstrom and Reimchen, 2000; Allenbach, 2011).

If important structures could not be seen very clearly on the scans the affected specimens were positioned under a stereomicroscope (Nikon, SMZ800) to set the landmarks correctly.

Microsoft Excel was used to calculate the mean and the standard deviation of the pelvic traits and of the standard length. Further statistical analysis was performed through IBM SPSS (version 24.0). A Kruskal-Wallis Test for independent samples was performed to detect if the differences between the left and the right side and between the populations are significant. The standard lengths of females and males of the populations BKD, FUB, T parents were also tested for significance. Each population was tested for pelvic differences between their left and right side. The BKD population demonstrated a

highly significant value ($p < 0.01$) and was not used for further investigation. The T 17 population also showed significant side differences but was expanded for exploration.

For the inter-population comparison a standardized value of the differences between the left and the right side was used by dividing the differences of the measured lengths through the mean of ABLL and ABLR. The chosen level of significance was $\alpha = 0.05$. To explore the influence of predatory pressure a comparison between FUB-AB (high-very low predatory pressure), FUB-HN (high-low predatory pressure) and HN-AB (low-very low predatory pressure) was drawn. To determine the effect of the different CO2 treatments the CO2 control group was compared to the CO2 high group, the CO2 parents to CO2 control and to CO2 high. The effects of temperatures were investigated by opposing T 17 with T 21 and T parents with T 17 and T 21. Habitat impacts were tested by comparing the stream populations HN with AB and LK with AB, and the lake population LK with the stream population FUB.

The graphs of the results were created with IBM SPSS (version 24.0) (boxplots) and Microsoft Excel (column chart).

Table 3: Description of the measured pelvic traits with their abbreviations and which scan (left, right or ventral) they are taken from. LM = landmark.

Abbreviation	**Description**	**Distances**	**Scanned side**
ABLL	ascending process length; left side	LM16-LM17	lateral left
ABLR	ascending process length; right side	LM16-LM17	lateral right
ABWL	ascending process width; left side	LM18-LM19	lateral left
ABWR	ascending process width; right side	LM18-LM19	lateral right
PSLL	pelvic spine length; left side	LM2-LM6	Ventral
PSLR	pelvic spine length; right side	LM1-LM5	Ventral
PPLL	posterior process length; left side	LM4-LM8	Ventral
PPLR	posterior process length; right side	LM3-LM7	Ventral
APLL	anterior process length; left side	LM6-LM13	Ventral
APLR	anterior process length; right side	LM5-LM12	Ventral
APWL	anterior process width; left side	LM10-LM11	Ventral
APWR	anterior process width; right side	LM9-LM11	Ventral
PPLFL	posterior process to midline of anterior processes; left side	LM4-LM15	Ventral
PPLFR	posterior process to midline of anterior processes ; right side	LM3- LM14	Ventral
FAPL	anterior process length from midline ; left side	LM13-LM15	Ventral
FAPR	anterior process length from midline ; right side	LM12- LM14	Ventral

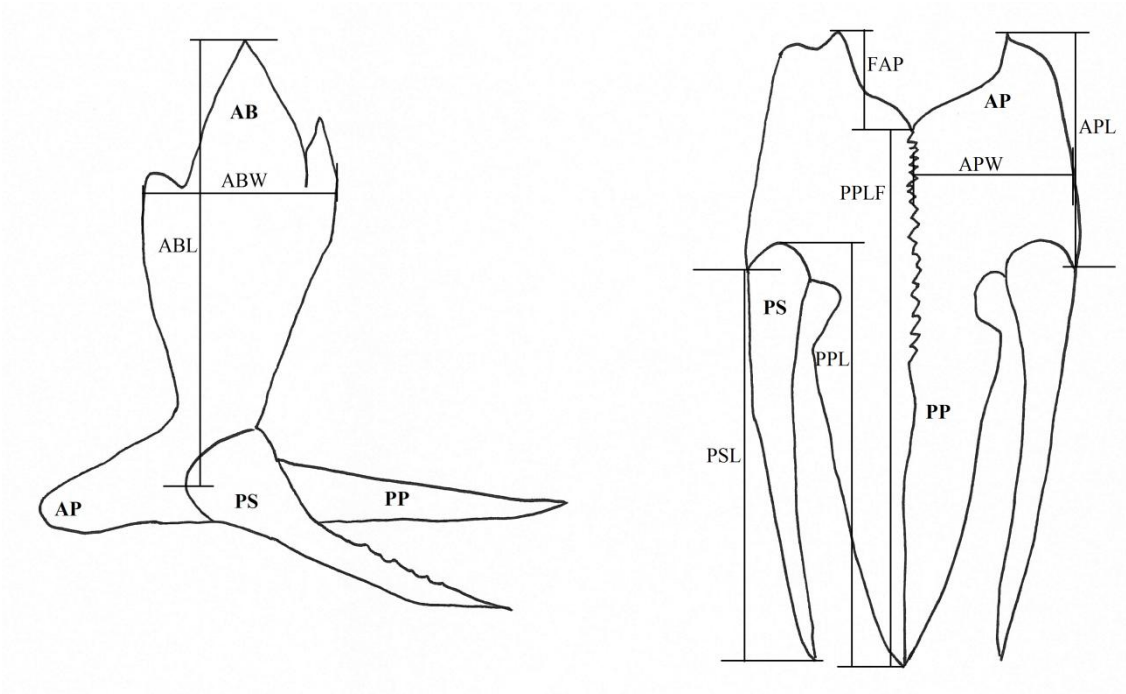

Figure 7: The pelvic complex of the threespine stickleback from a lateral (left) and ventral view (right). The anterior process (AP), the posterior process (PP) and the pelvic spine (PS).The measured pelvic traits (modified after Klepaker *at al*. 2012): ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3. Results

3.1. Size of all populations (Fig. 8, Table 4)

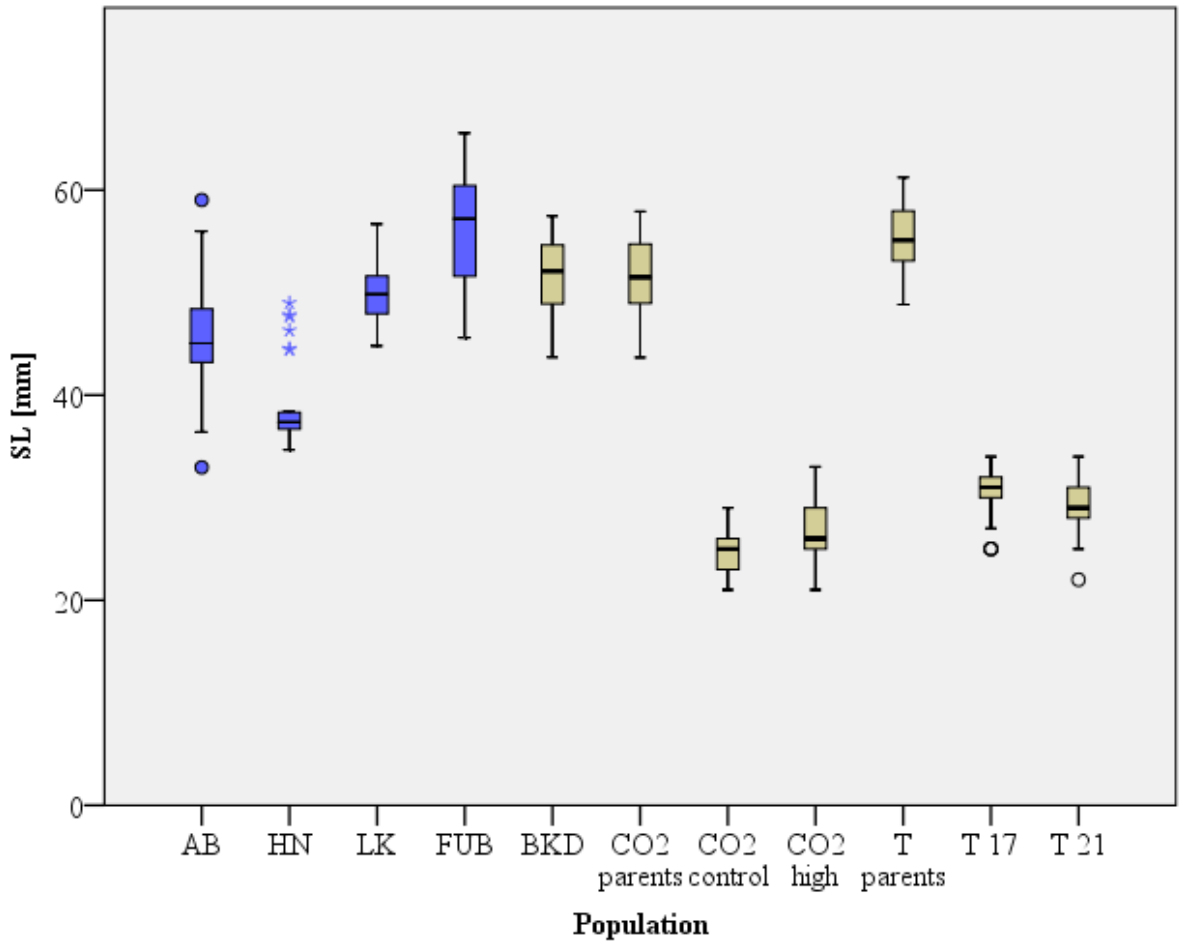

Figure 8: Size (SL [mm]) of all populations. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Freshwater populations are expressed in blue. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

The largest freshwater population was FUB, the smallest HN. The largest oceanic population was the temperature parents. The BKD population and the CO2 parents were of similar size. From the juveniles the temperature specimens were larger than the CO2 specimens.

From the adult freshwater populations the HN had the smallest variation in size whereas size variation was very distinct in AB. In general, the lake population (FUB) was larger than the stream populations (AB, HN, LK). From the oceanic populations T parents were somewhat larger than BKD and CO2 which were both of similar size. Both juveniles raised under different temperatures were larger than

the specimens raised under different CO2 levels. Within the temperature juveniles T 17 were larger than T 21 and CO2 high were somewhat larger than CO2 control.

3.2. Sexual dimorphism in size (Fig. 9, Table 5)

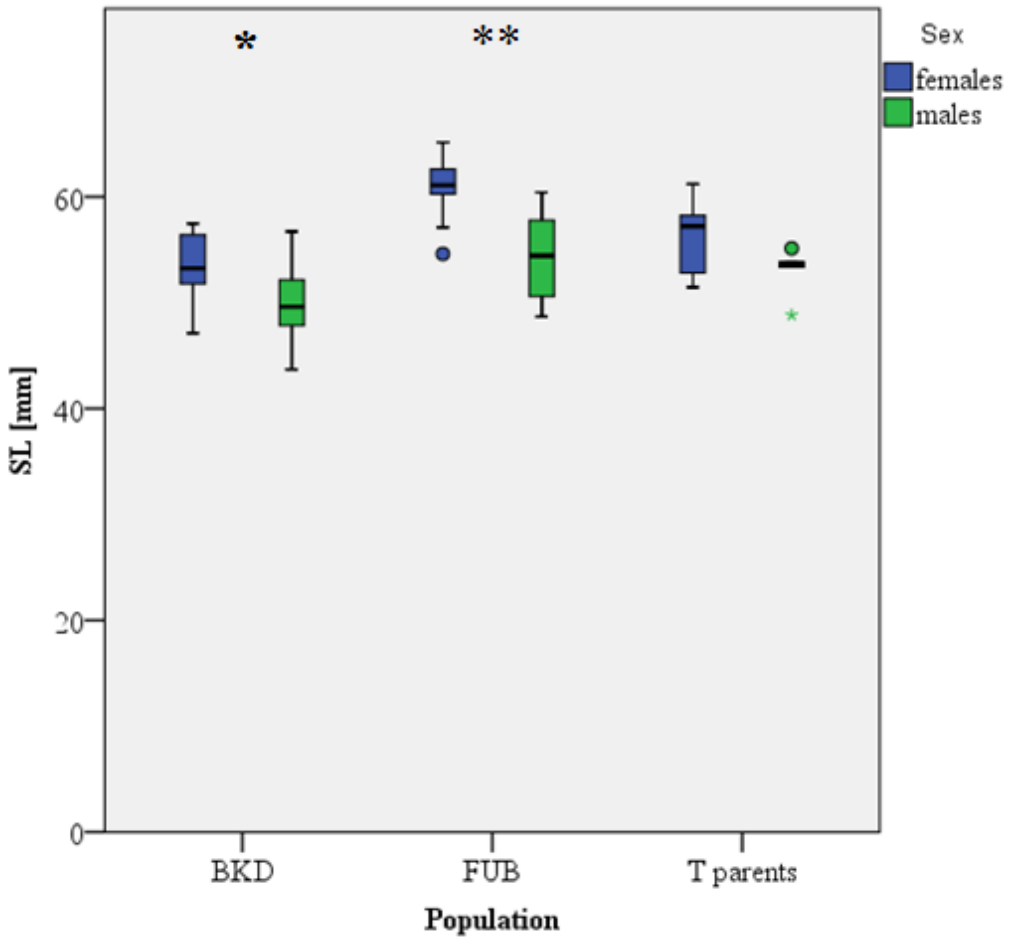

Figure 9: Size (SL [mm]) of females (blue) and males (green) of the populations BKD, FUB, T parents. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). BKD = Brede Å system, Kisbaek creek; FUB = Fussacher Bay; T parents = marine adults from Sylt-Rømø Bight.

Table 4: Standard length (SL) range [mm], Mean [mm] and Standard deviation (SD) [mm] of the populations. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Population	SL range [mm]	Mean [mm]	SD [mm]
AB	32.96 - 59.03	45.30	5.27

HN	34.66 - 48.99	38.70	3.87
LK	44.79 - 56.69	50.00	3.00
FUB	45.59 - 65.55	56.56	5.53
BKD	43.70 - 57.46	51.54	3.62
CO2 parents	43.67 - 57.91	51.38	4.23
CO2 control	21.00 - 29.00	25.00	2.08
CO2 high	21.00 - 33.00	27.00	2.54
T parents	48.84 - 61.22	55.24	3.50
T 17	25.00 - 34.00	30.68	1.91
T 21	22.00 - 34.00	29.41	2.25

Females were in general larger than males in the three populations with determined sex. Most distinct size differences were found in the lake population (FUB) whereas the size differences between the sexes were similar in the both oceanic populations (BKD, T parents). Females were significantly larger in the BKD ($p < 0.05$) and highly significant in the FUB ($p < 0.01$) population. The T parents did not show significant differences between sexes.

Table 5: The Standard length (SL) range [mm], Mean [mm] and Standard deviation (SD) [mm] of the populations with determined sex. Total = all specimens of the population. Males–females = FUB specimens with determined sex. BKD = Brede Å system, Kisbaek creek; FUB = Fussacher Bay; T parents = marine adults from Sylt-Rømø Bight.

Population	**SL range [mm]**	**Mean [mm]**	**SD [mm]**
BKD total	43.70 – 57.46	51.54	3.62
BKD males	43.70 – 56.72	49.73	3.27
BKD females	47.12 – 57.46	53.34	3.07
T parents total	48.84 – 61.22	55.24	3.50
T parents males	48.84 – 55.13	52.97	2.41
T parents females	51.45 – 61.22	56.38	3.48
FUB males-females	45.59 – 65.55	57.74	4.78
FUB males	48.69 – 60.42	54.40	4.02
FUB females	48.69 -65.15	60.82	3.06

3.3. Pelvic trait measurements

3.3.1. Left-right comparison

The results revealed that there are generally differences between the left and the right side of adult sticklebacks in the measured traits regardless of habitat (lake, stream or oceanic), predatory pressure (AB, HN, FUB and BKD) or environmental stressors (temperature, CO2) (Table 6, Table 7; Table 10; Table 11). Nevertheless, these differences between left and right side were not significant in most of the cases except for one trait (APW) in one population (BKD) and in the temperature experiment (T 17): BKD (APW, $p < 0.01$) and T 17 (APW, $p < 0.05$).

Table 6: The Mean [mm] of the pelvic traits of all populations measured from the lateral side. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABLL = ascending process length, left side; ABLR = ascending process length , right side; ABWL = ascending process width, left side; ABWR = ascending process width, right side.

Population	**ABLL**	**ABLR**	**ABWL**	**ABWR**
AB	6.20	6.13	2.52	2.62
HN	5.18	5.28	2.23	2.21
LK	7.67	7.65	3.41	3.11
FUB	8.61	8.76	3.81	4.08
BKD	6.86	6.98	2.98	3.14
CO2 parents	6.47	6.55	2.88	3.00
CO2 control	3.06	3.01	1.16	1.18
CO2 high	3.30	3.23	1.34	1.29
T parents	7.06	7.22	3.34	3.34
T 17	3.84	3.90	1.55	1.51
T 21	3.55	3.60	1.51	1.46

Table 7: The Mean [mm] of the pelvic traits of all populations measured from the ventral side. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. APLL = anterior process length, left side; APLR = anterior process length, right side; APWL = anterior process width, left side; APWR = anterior process width, right side; FAPL = anterior process length from midline, left side; FAPR = anterior process length from midline, right side; PPLL = posterior process length, left side; PPLR = posterior process length, right side; PPLFL = posterior process to midline of anterior processes, left side; PPLFR = posterior process to midline of anterior processes, right side; PSLL = pelvic spine length, left side; PSLR = pelvic spine length, right side.

.

Population	**APLL**	**APLR**	**APWL**	**APWR**	**FAPL**	**FAPR**	**PPLL**	**PPLR**	**PPLFL**	**PPLFR**	**PSLL**	**PSLR**
AB	3.68	3.66	2.70	2.72	1.90	1.89	6.69	6.66	8.24	8.23	5.16	5.29
HN	3.23	3.21	2.15	2.17	1.43	1.41	6.24	6.25	7.90	7.90	5.29	5.34
LK	4.60	4.57	3.04	3.07	0.64	0.63	9.41	9.44	12.09	12.09	7.63	7.79
FUB	5.15	5.12	3.49	3.24	0.58	0.47	10.90	10.90	13.88	13.88	8.35	8.42
BKD	4.36	4.24	2.79	2.57	0.54	0.46	9.34	9.35	11.96	11.95	8.40	8.46
CO2 parents	4.61	4.41	2.77	2.60	0.55	0.65	9.89	9.83	12.58	12.57	8.70	8.60

CO2 control	-	-	-	-	-	-	-	-	-	-	4.67	4.65
CO2 high	-	-	-	-	-	-	-	-	-	-	5.11	5.14
T parents	4.78	4.60	3.18	2.93	0.62	0.60	13.13	13.13	10.47	10.45	9.15	9.32
T 17	2.62	2.58	1.57	1.51	0.70	0.65	5.19	5.12	6.82	6.82	5.95	5.88
T 21	2.51	2.44	1.53	1.50	0.71	0.69	4.70	4.65	6.19	6.18	5.52	5.49

The adult freshwater populations HN and AB revealed the smallest values in all pelvic traits and FUB was largest in all traits (Table 6; Table 7). The smallest juvenile population in pelvic traits was CO2 control, the largest was T 17.

Table 8: The Median [mm] of the pelvic traits of all populations measured from the lateral side. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABLL = ascending process length, left side; ABLR = ascending process length , right side; ABWL = ascending process width, left side; ABWR = ascending process width, right side.

Population	ABLL	ABLR	ABWL	ABWR
AB	6.16	6.19	2.47	2.66
HN	5.14	5.12	2.15	2.22
LK	7.45	7.66	3.05	2.98
FUB	8.68	8.74	3.77	3.79
BKD	6.87	7.05	2.98	3.17
CO2 parents	6.45	6.66	2.88	2.98
CO2 cont	3.03	3.05	1.18	1.15
CO2 high	3.28	3.21	1.35	1.30
T parents	7.08	7.24	3.41	3.46
T 17	3.86	3.90	1.51	1.47
T 21	3.54	3.64	1.48	1.45

Table 9: The Median [mm] of the pelvic traits of all populations measured from the ventral side. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. APLL = anterior process length, left side; APLR = anterior process length, right side; APWL = anterior process width, left side; APWR = anterior process width, right side; FAPL = anterior process length from midline, left side; FAPR = anterior process length from midline, right side; PPLL = posterior process length, left side; PPLR = posterior process length, right side; PPLFL = pos-

terior process to midline of anterior processes, left side; PPLFR = posterior process to midline of anterior processes, right side; PSLL = pelvic spine length, left side; PSLR = pelvic spine length, right side.

Population	APL L	APL R	APW L	APW R	FAP L	FAP R	PPL L	PPL R	PPLF L	PPLF R	PSL L	PSL R
AB	3.72	3.58	2.70	2.69	1.85	1.81	6.63	6.58	8.27	8.19	5.17	5.21
HN	3.17	3.15	2.03	2.05	1.36	1.31	6.18	6.12	7.61	7.63	5.25	5.20
LK	4.59	4.59	3.03	3.01	0.63	0.51	9.52	9.54	12.17	12.16	7.62	7.99
FUB	5.13	4.97	3.53	3.11	0.53	0.44	11.46	11.08	13.98	13.98	8.26	8.33
BKD	4.36	4.19	2.76	2.59	0.32	0.21	9.25	9.32	11.94	11.94	8.36	8.45
CO2 parents	4.61	4.49	2.85	2.56	0.28	0.56	9.94	9.92	12.82	12.82	8.79	8.57
CO2 cont	-	-	-	-	-	-	-	-	-	-	4.61	4.73
CO2 high	-	-	-	-	-	-	-	-	-	-	5.15	5.16
T parents	4.87	4.89	3.28	2.86	0.37	0.43	10.59	10.45	13.45	13.45	9.16	9.31
T 17	2.65	2.58	1.58	1.54	0.80	0.75	5.30	5.23	6.87	6.86	5.94	5.87
T 21	2.51	2.42	1.54	1.49	0.72	0.74	4.68	4.64	6.18	6.17	5.49	5.44

3.3.1.1. AB

The AB population showed slight differences in the length of all eight investigated traits with APL, APW, FAP, PPLF, PPL longer on the left side and ABL, ABW and PSL longer on the right side (Fig. 10).

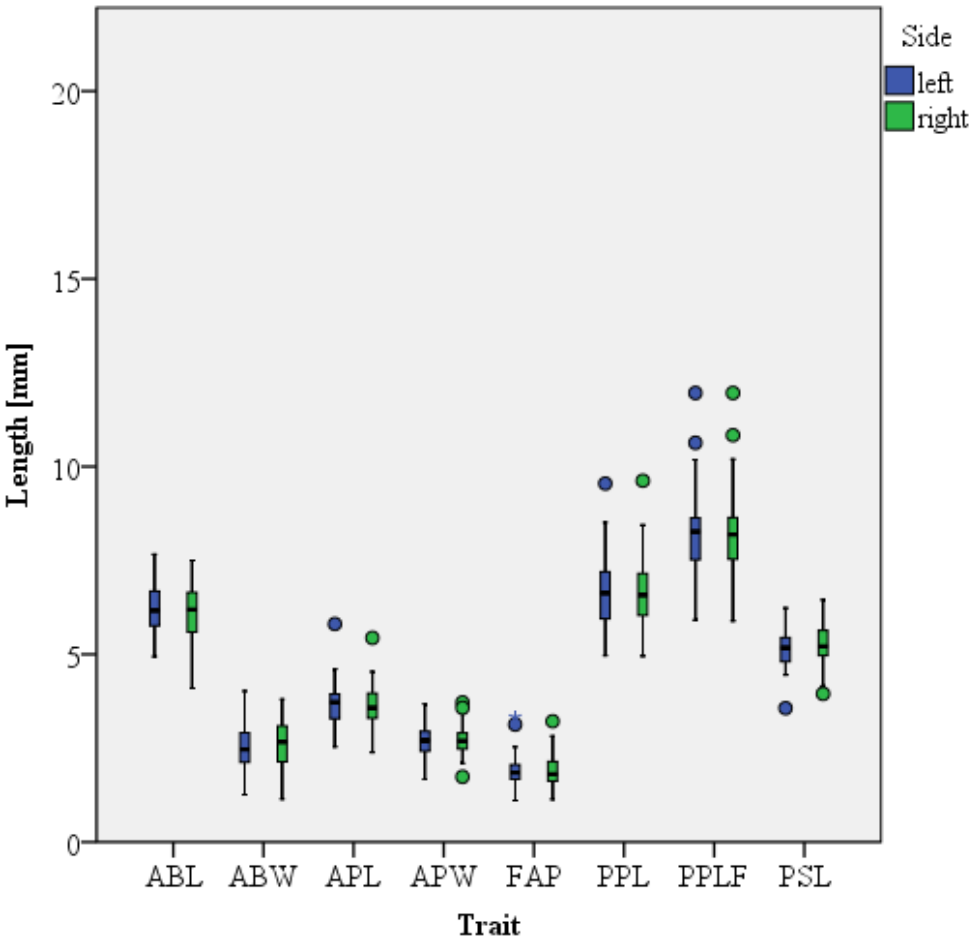

Figure 10: Length of pelvic traits [mm] on both sides of AB. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. AB =Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.1.2. HN

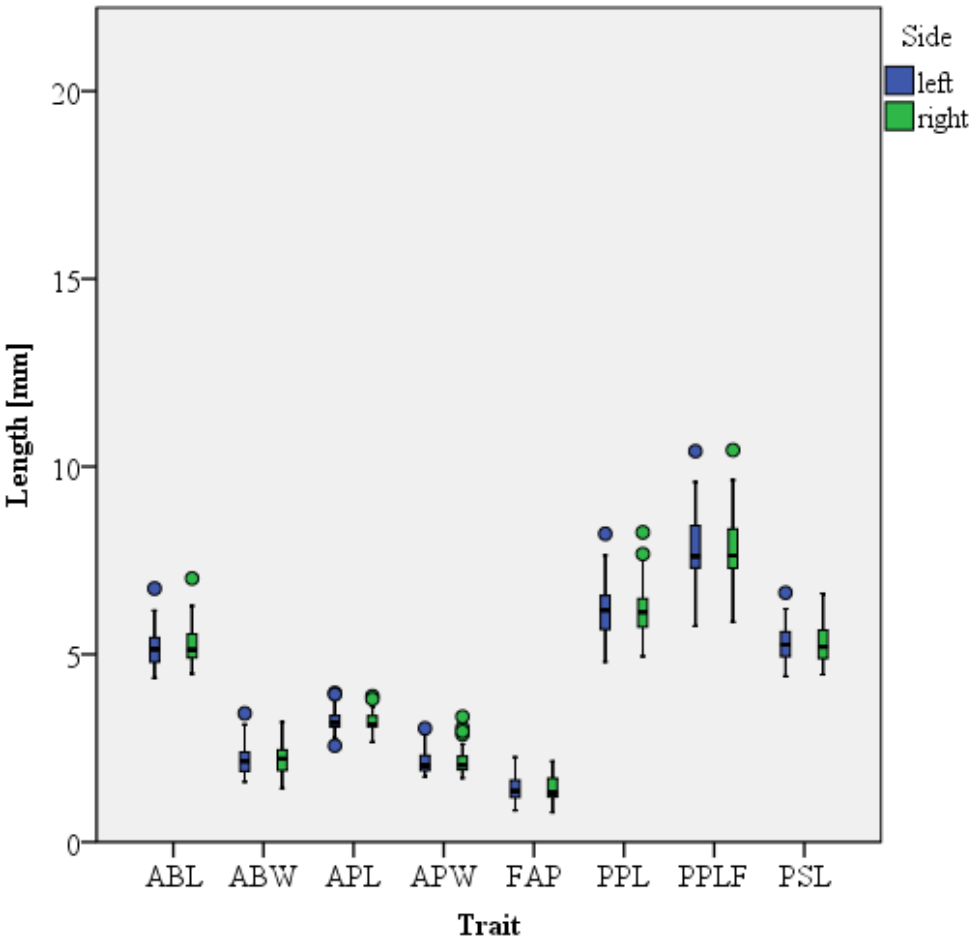

Figure 11: Length of pelvic traits [mm] on both sides of HN. Black lines mark the median. Circles mark single outliers. HN = Neubach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The HN population showed slight differences in the length of all eight investigated traits with ABL, APL, FAP, PPL and PSL longer on the left side and ABW, APW and PPLF longer on the right side (Fig. 11).

3.3.1.3. LK

The LK population showed slight differences in the length of seven of the investigated eight traits with the left side longer in ABW, APW, FAP, PPLF and ABL, PPL and PSL longer on the right side (Fig. 12). The trait APL presented the same length on both sides.

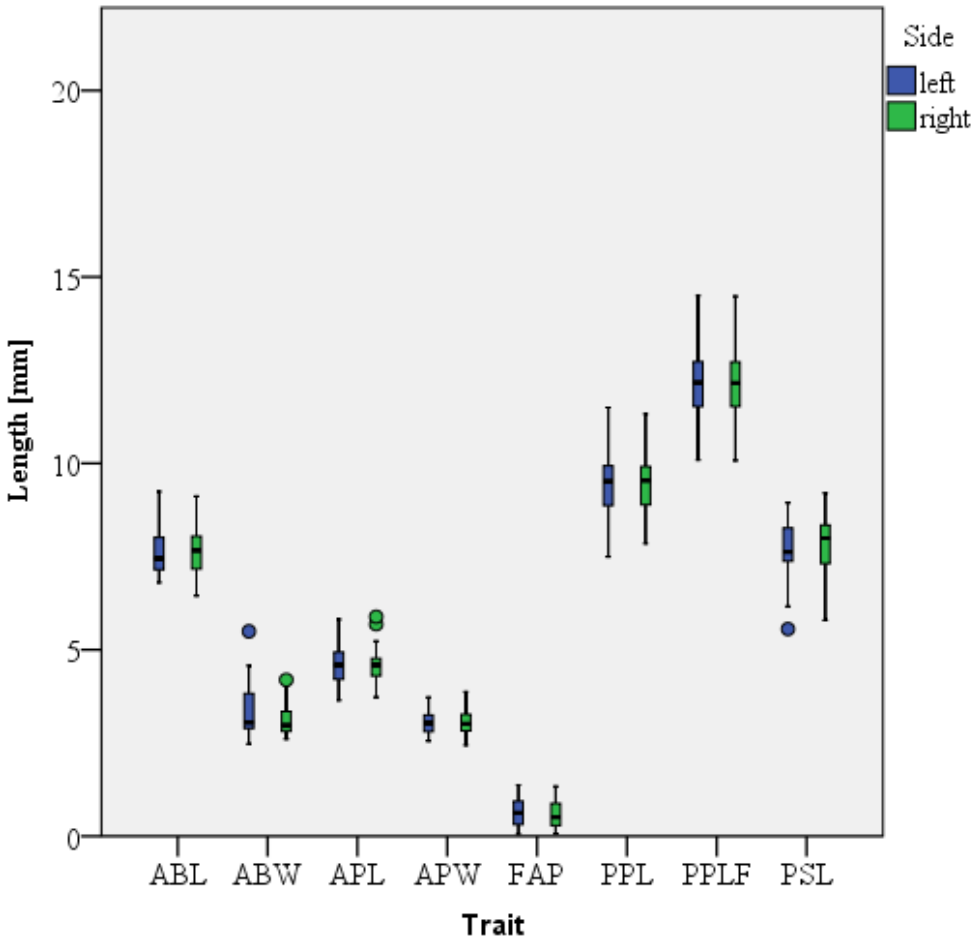

Figure 12: Length of pelvic traits [mm] on both sides of LK. Black lines mark the median. Circles mark single outliers. LK = Lustenauer Kanal. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.1.4. FUB

The FUB population showed slight differences in the length of seven of the eight investigated traits with APL, APW, FAP, PPL longer on the left side and ABL, ABW and PSL were longer on the right side (Fig. 13). PPLF did not differ in length between the sides.

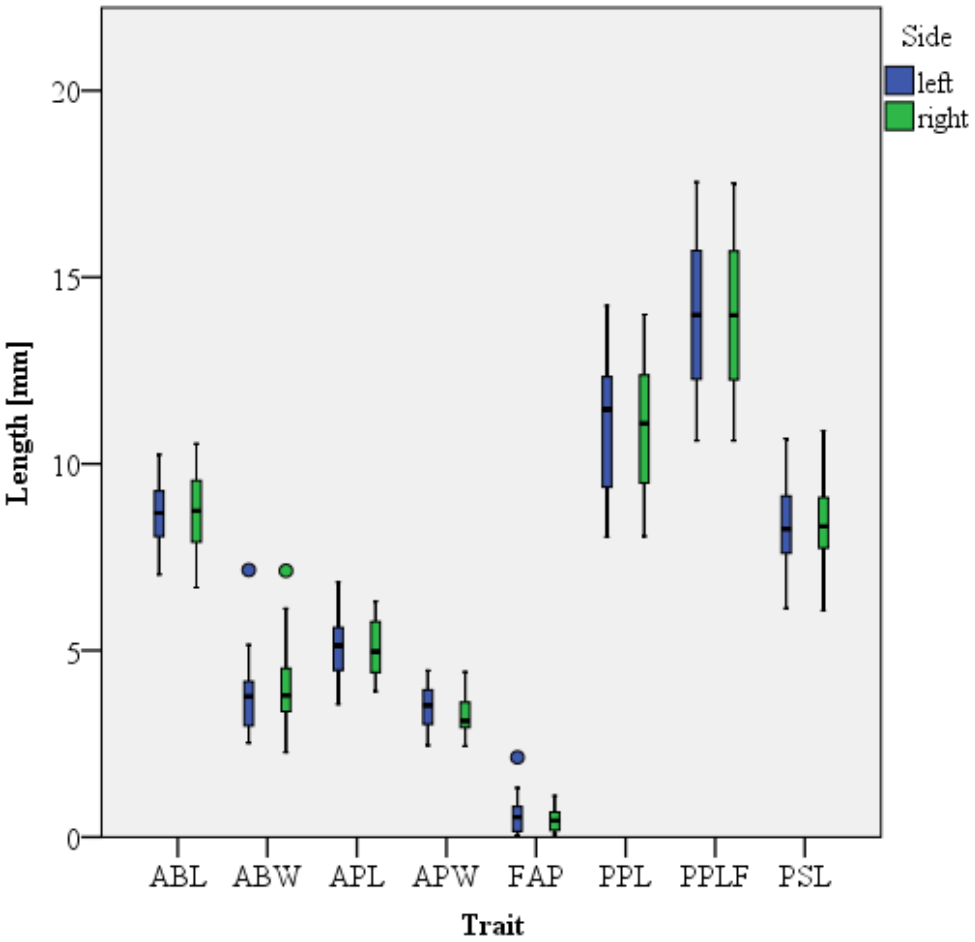

Figure 13: Length of pelvic traits [mm] on both sides of FUB. Black lines mark the median. Circles mark single outliers. FUB = Fussacher Bay. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.1.5. BKD

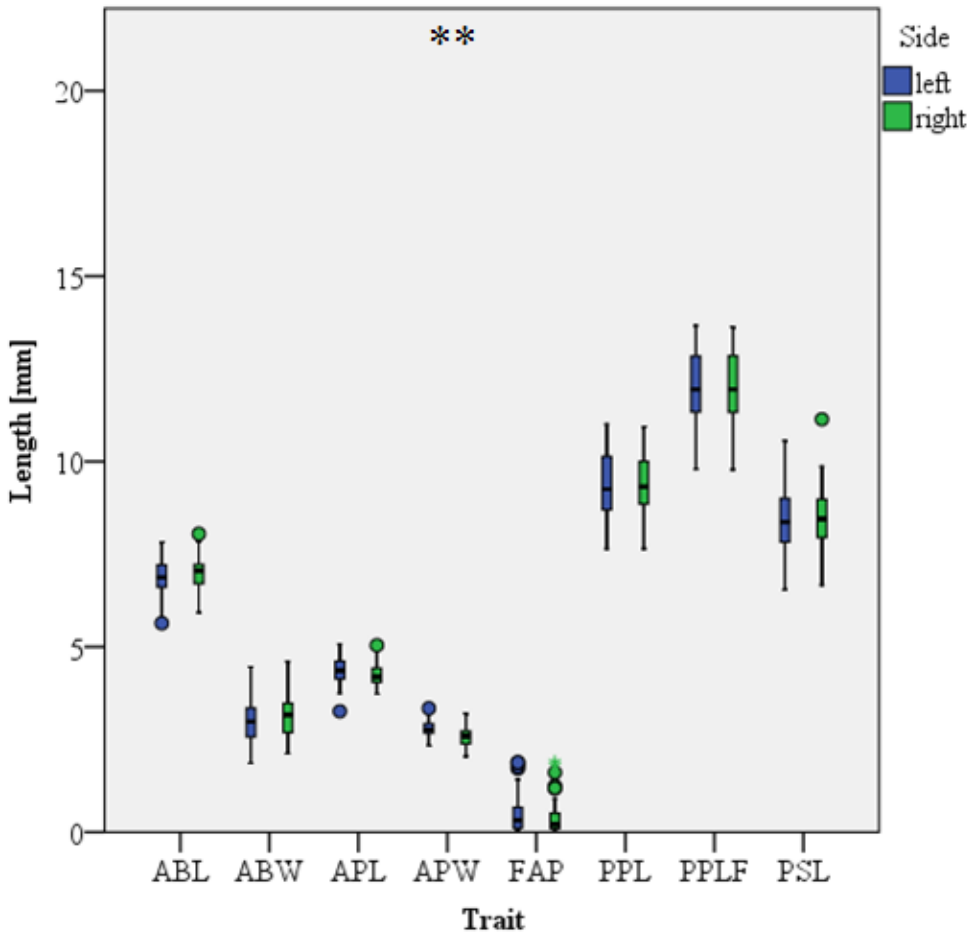

Figure 14: Length of pelvic traits [mm] on both sides of BKD. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$). BKD = Brede Å system, Kisbaek creek. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The BKD population showed slight differences in the length of seven of eight investigated traits with ABL, ABW, PPL and PSL longer on the right side and APL, APW and FAP longer on the left side (Fig. 14). PPLF did not differ in length between the sides. APW displayed a significant value ($p < 0.01$).

3.3.1.6. CO2 parents

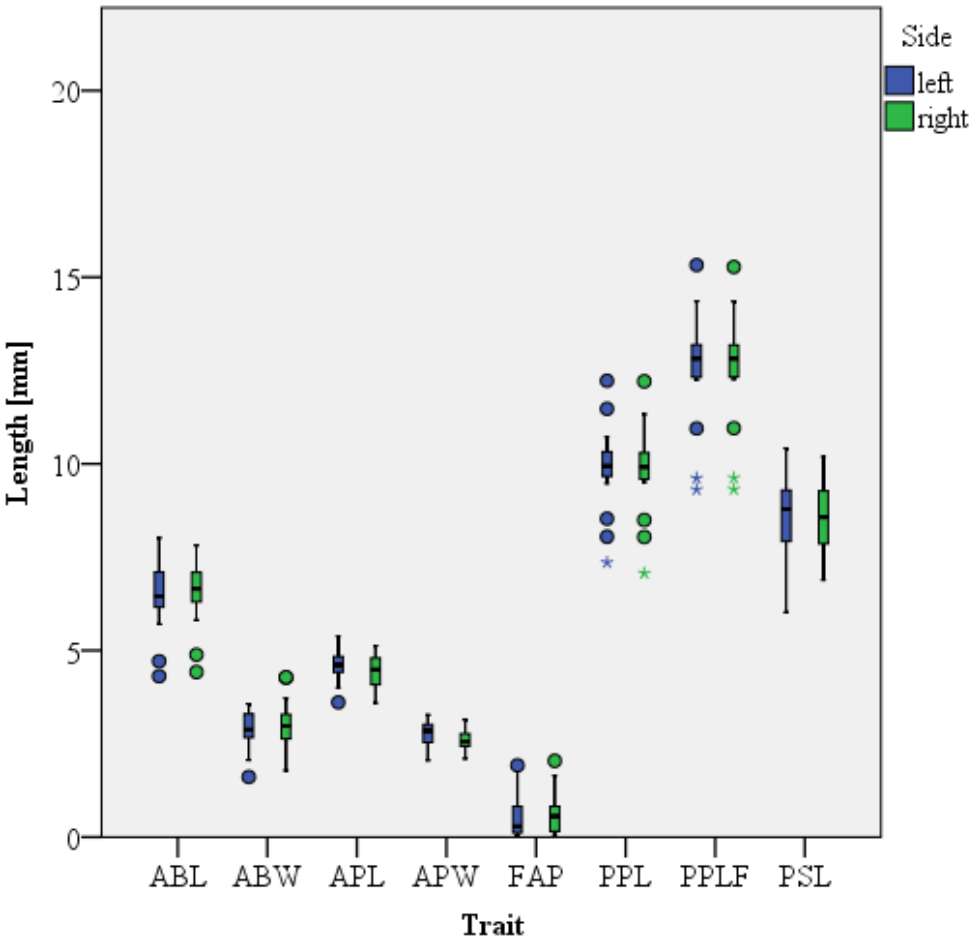

Figure 15: Length of pelvic traits [mm] on both sides of CO2 parents. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. CO2 parents = marine adults from Sylt-Rømø Bight. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The CO2 parents population revealed slight differences in seven from eight investigated traits with APL, APW, PPL and PSL longer on the left side and ABL, ABW and FAP longer on the right (Fig. 15). PPLF did not differ in length between the sides.

3.3.1.7. CO2 control

The specimens of CO2 control showed slight differences in the length of all three investigated traits with ABL and PSL longer on the right side and ABW longer on the left side (Fig. 16).

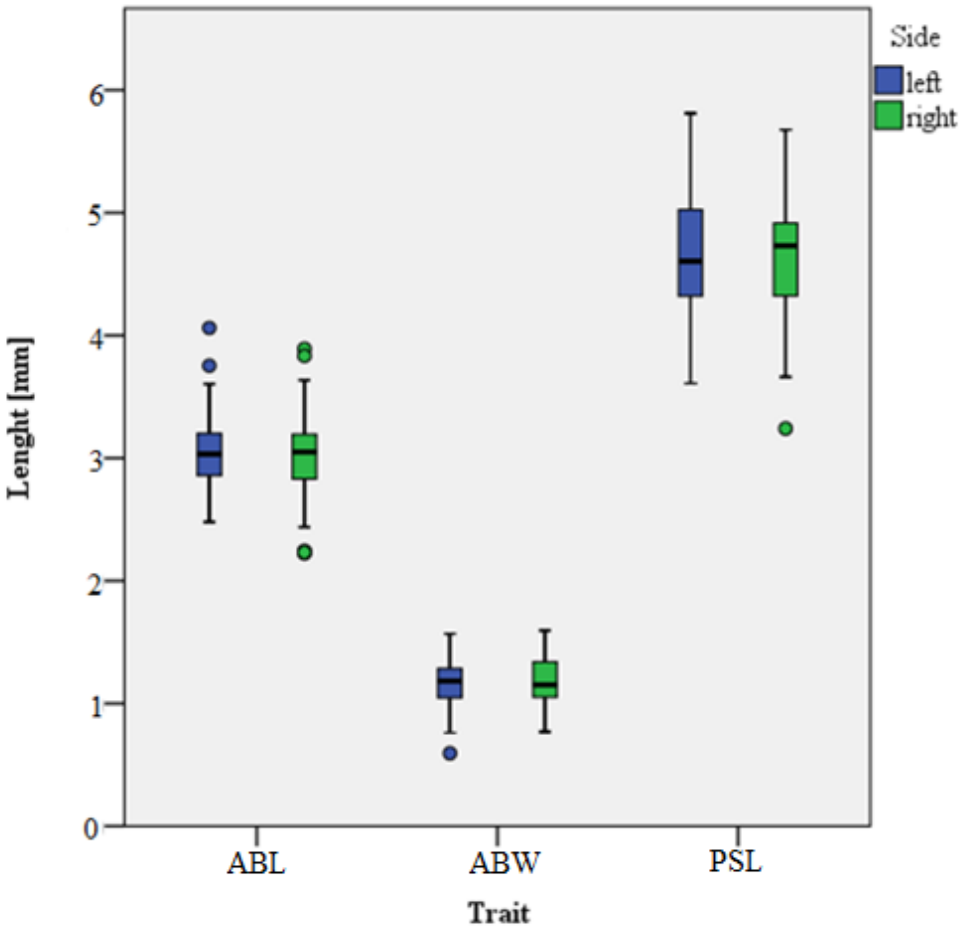

Figure 16: Length of pelvic traits [mm] on both sides of CO2 control. Black lines mark the median. Circles mark single outliers. CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions. ABL = ascending process length, ABW = ascending process width, PSL = pelvic spine length.

3.3.1.8. CO2 high

The CO2 high population showed slight differences in all three investigated traits with ABL, ABW longer on the left side and PSL longer on the right side (Fig. 17).

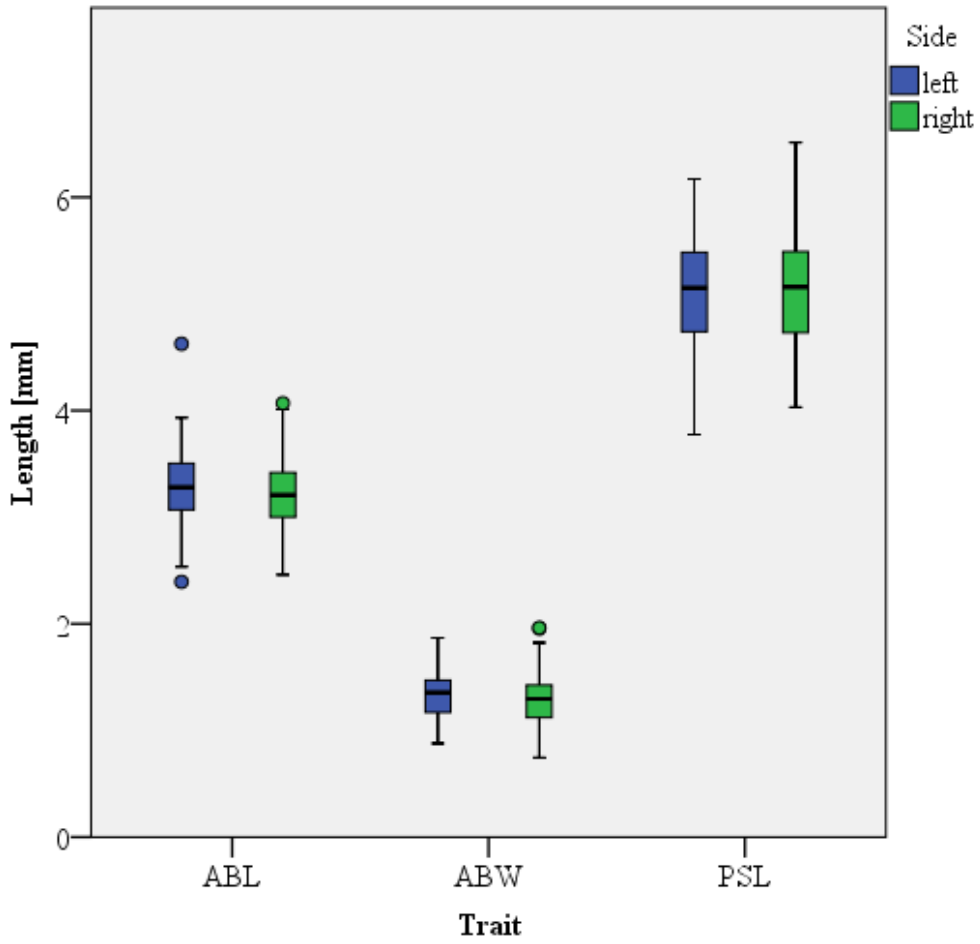

Figure 17: Length of pelvic traits [mm] on both sides of CO2 high. Black lines mark the median. Circles mark single outliers. CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions. ABL = ascending process length, ABW = ascending process width, PSL = pelvic spine length.

3.3.1.9. T parents

The T parents population showed slight differences in the length of seven of the eight investigated traits with APW and PPL longer on the left side and ABL, ABW, APL, FAP and PSL longer on the right side (Fig. 18). The trait PPLF had the same length on both sides.

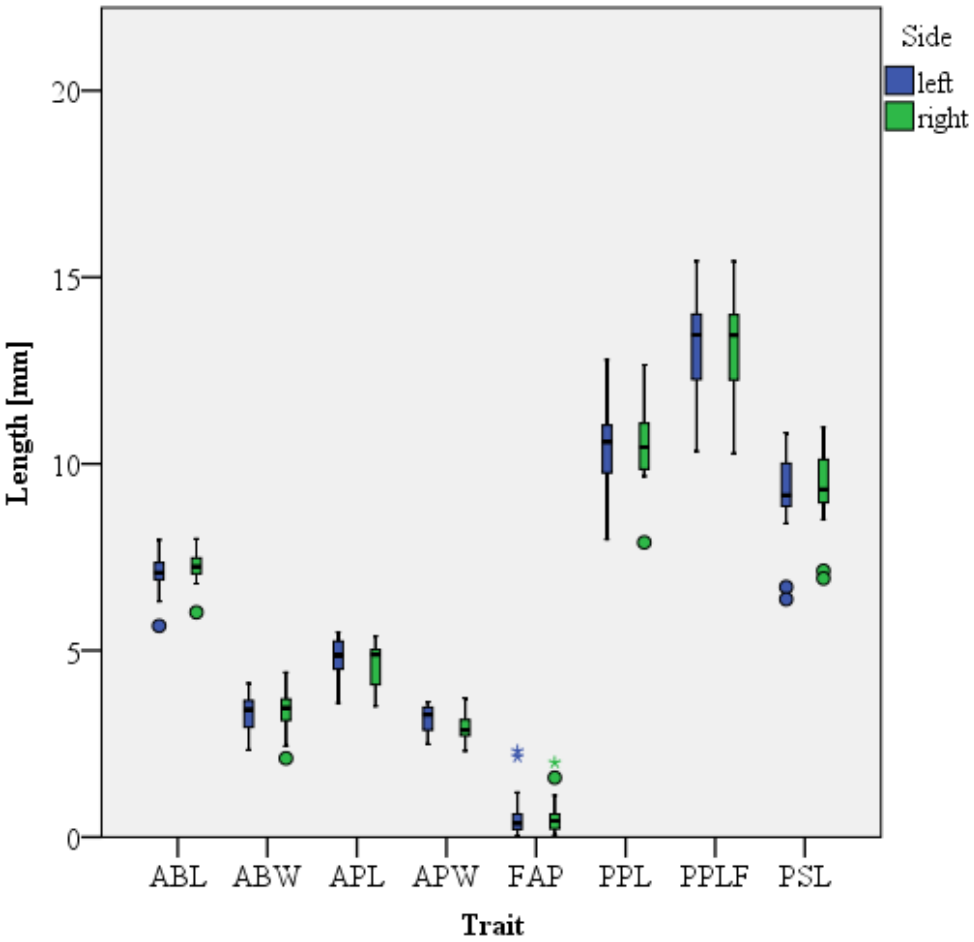

Figure 18: Length of pelvic traits [mm] on both sides of T parents. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. T parents = marine adults from Sylt-Rømø Bight. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.1.10. T 17

The T 17 population showed slight differences in the lengths of all eight investigated traits with ABW, APL, APW, FAP, PPLF, PPL and PSL longer on their left side and ABL longer on the right side (Fig. 19). APW ($p < 0.05$) revealed significant differences between the lateral sides.

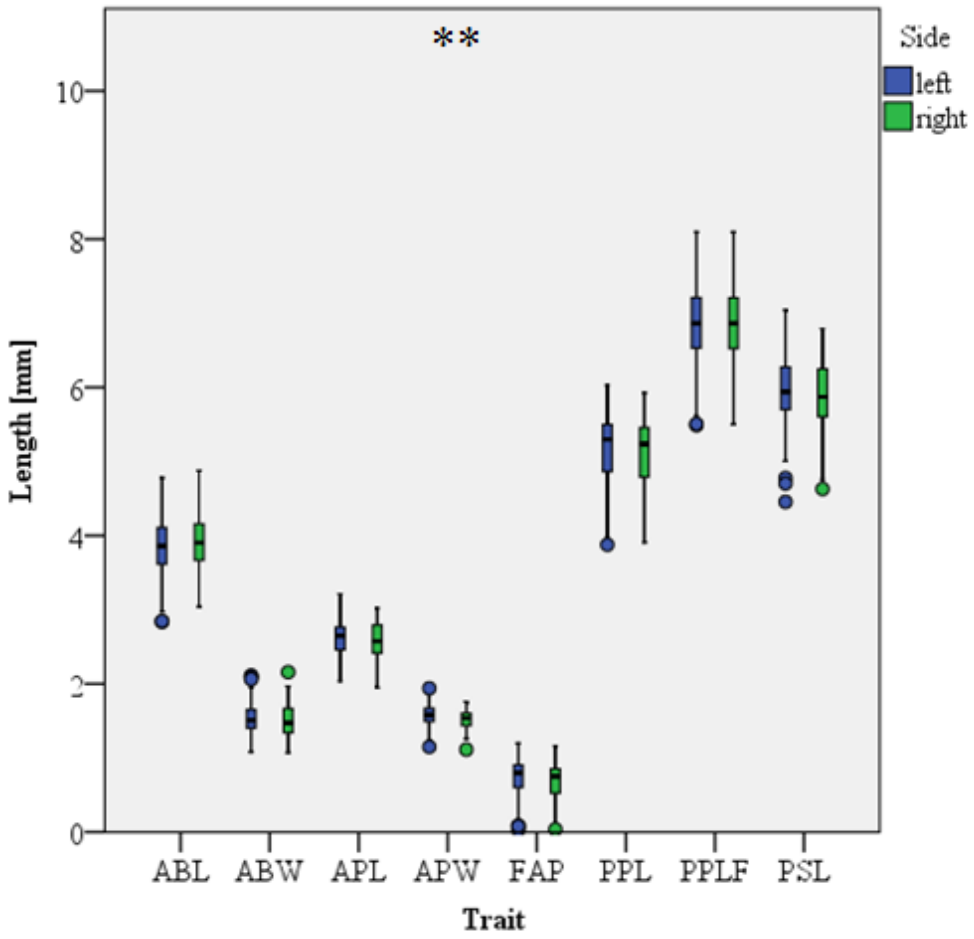

Figure 19: Length of pelvic traits [mm] on both sides of T 17. Black lines mark the median. Circles mark single outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.1.11. T 21

The T 21 population showed slight differences in the length of all eight traits with ABW, APL, APW, PPLF, PPL and PSL longer on the left and ABL and FAP longer on the right (Fig. 20).

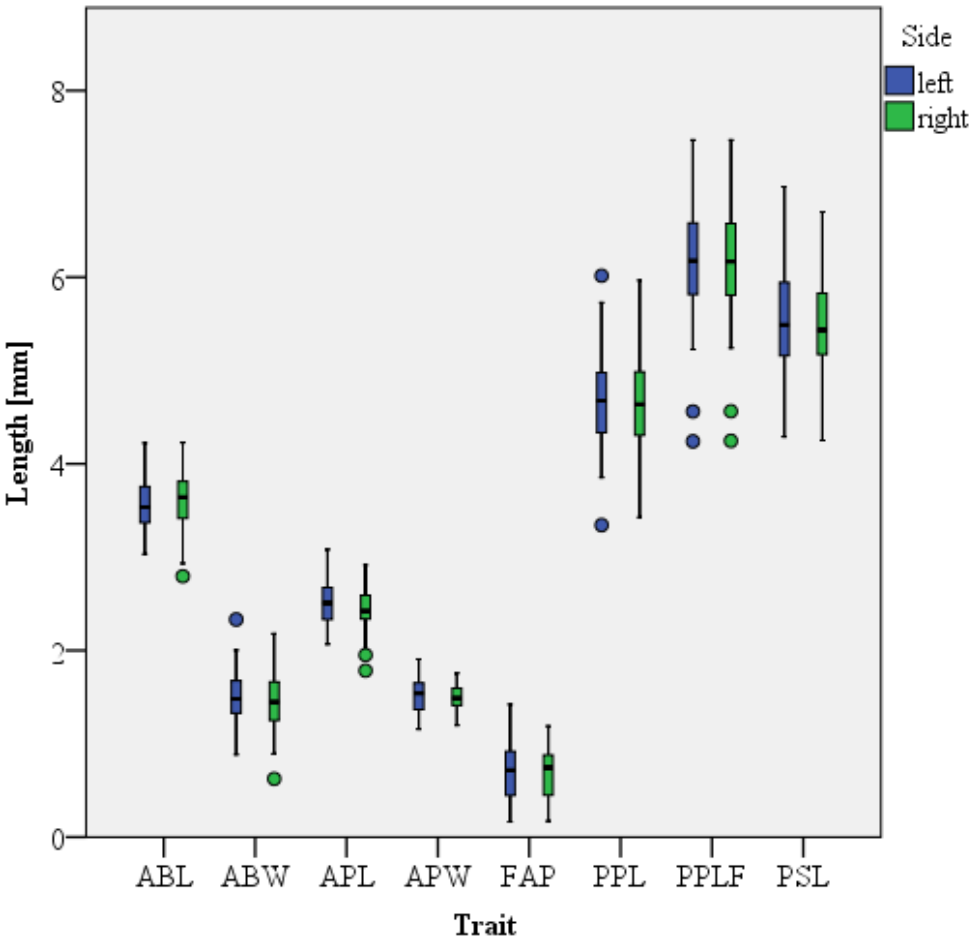

Figure 20: Length of pelvic traits [mm] on both sides of T 21. Black lines mark the median. Circles mark single outliers. T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.2. Inter-population comparison

3.3.2.1. Predatory pressure

3.3.2.1.1. FUB-AB

The comparison of FUB-AB revealed three significant to very significant values concerning ABW ($p < 0.01$), FAP ($p < 0.01$), PPLF ($p < 0.001$).

Table 10: Mean difference between the sides [%] to ABL (ascending process length) from the lateral scans in all populations. ABL = ascending process length. ABW = ascending process width. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a tem-

perature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Population	% Difference ABL to ABL [%]	% Difference ABW to ABL [%]
HN	0.94	0.97
AB	1.04	0.89
LK	0.68	0.98
FUB	0.63	1.18
BKD	0.48	0.63
CO2 parents	0.60	1.17
CO2 control	2.42	1.97
CO2 high	2.10	1.37
T parents	0.54	0.60
T 17	1.44	0.96
T 21	1.63	1.64

Table 11: Mean difference (Diff) between the sides [%] to ABL (ascending process length) from the ventral scans in all populations. APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Population	**% Diff APL to ABL [%]**	**% Diff APW to ABL [%]**	**% Diff FAP to ABL [%]**	**% Diff PPL to ABL [%]**	**% Diff PPLF to ABL [%]**	**% Diff PSL to ABL [%]**
HN	0.11	0.59	0.38	0.43	0.16	0.78
AB	0.73	0.44	0.68	0.36	0.14	0.79
LK	0.24	0.27	0.38	0.37	0.03	0.57
FUB	0.34	0.39	0.35	0.23	0.03	0.41
BKD	0.44	0.55	0.39	0.20	0.04	0.58
CO2 parents	0.64	0.52	0.63	0.21	0.04	0.71
CO2 control	-	-	-	-	-	1.29
CO2 high	-	-	-	-	-	1.49
T parents	0.54	0.67	0.35	0.23	0.04	0.69
T 17	0.87	0.94	0.76	0.68	0.08	1.00
T 21	1.07	1.12	0.88	0.77	0.07	1.49

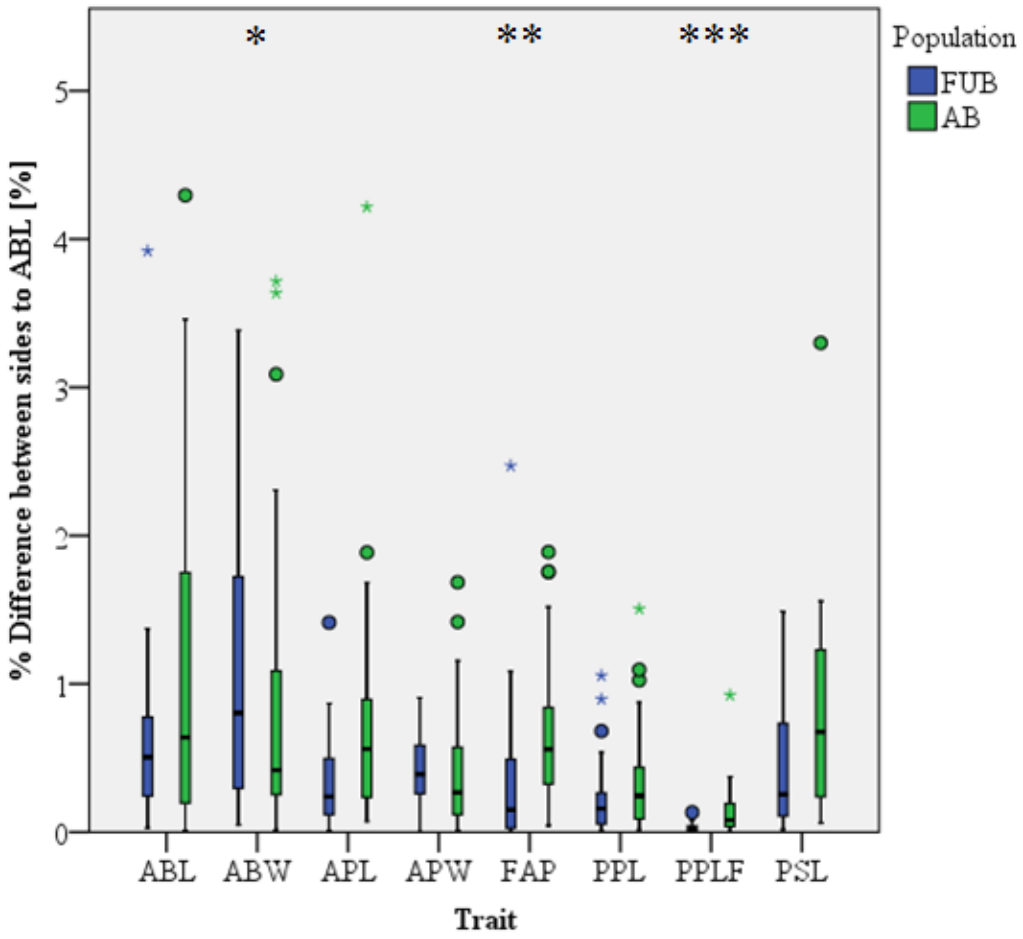

Figure 21: The difference between the left and the right side [%] to the distance ABL (ascending process length) of the FUB population compared to the AB population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). FUB = Fussacher Bay; AB = Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

ABL, ABW and PSL were longer on the right side and APL, APW, FAP and PPL were longer on the left side in both populations. PPLF did not differ in FUB and differed only very little in the AB population with the left side being slightly longer.

The difference between the lateral sides was higher in AB in six of eight investigated traits with ABL, APL, FAP, PPL, PPLF and PSL compared to FUB (Fig. 21). FUB was more asymmetric in the two traits ABW and APW.

3.3.2.1.2. FUB-HN

The significant differences between FUB – HN were ABW ($p < 0.05$), APL ($p < 0.001$) and PPLF ($p < 0.001$) (Fig. 22). APL, FAP and PPL were longer on the left side in both populations. PPLF had the same length on both sides in FUB and was slightly longer on the right side in HN. ABL was longer on the left side, APW longer on the right side, PSL longer on the left side in HN in contrast to FUB. FUB

had no differences between the lateral sides in the length PPLF. HN fish showed higher values on the left side concerning this trait.

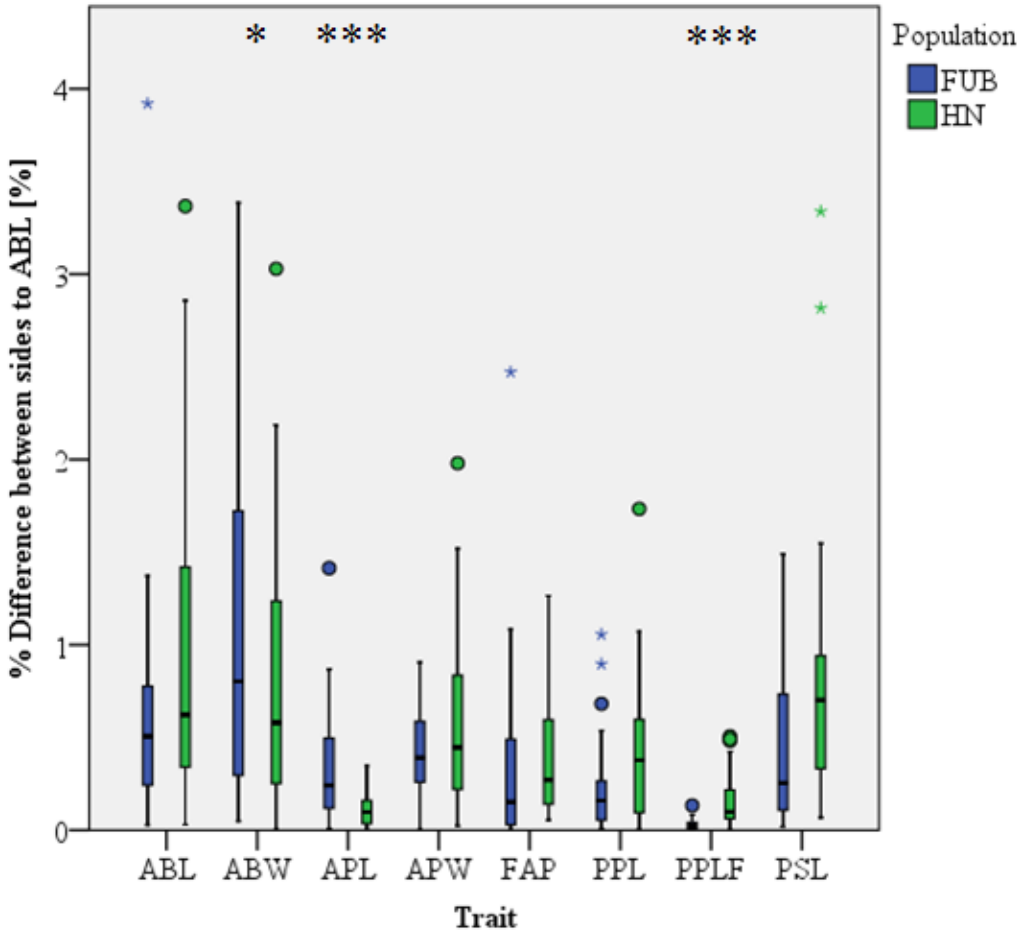

Figure 22: The difference between the left and the right side [%] to the distance ABL (ascending process length) of the FUB population compared to the HN population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). FUB = Fussacher Bay; HN = Neubach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The HN population revealed greater differences between the lateral sides in six of eight investigated traits with ABL, APW, FAP, PPL, PPLF and PSL compared to FUB (Fig. 22). FUB was more asymmetric in the two traits ABW, APL.

3.3.2.1.3. HN-AB

HN compared to AB showed highly significant values for APL ($p < 0.001$) and FAP ($p < 0.001$) (Fig. 23). APL, FAP, PPL were longer on the left side in both populations and ABW was longer on the right side. ABL in AB was longer on the right side and longer on the left side in the specimens from HN. APW and PPLF were longer on the left side in AB in contrast to HN. PSL was shorter on the left side in AB and longer on the left side in HN.

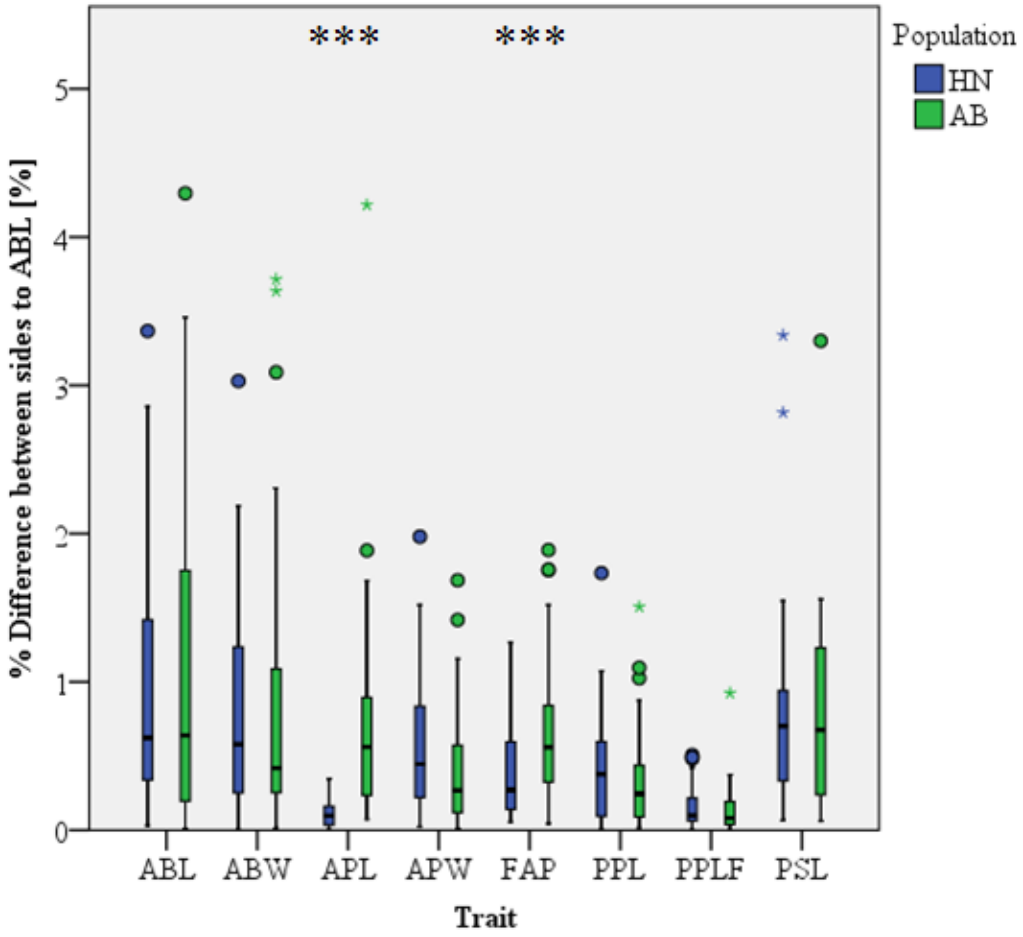

Figure 23: The difference between the left and the right side [%] to the distance ABL (ascending branch length) of the HN population compared to the AB population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HN = Neubach; AB = Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The HN population showed greater differences between the lateral sides in five of eight investigated traits with ABW, APW, PPL, PPLF and PSL compared to AB. The three traits ABL, APL and FAP demonstrated more asymmetries in AB.

3.3.2.2. Habitat

3.3.2.2.1. HN-AB

The HN-AB comparison revealed two highly significant values for APL ($p < 0.001$) and FAP ($p < 0.001$ (Fig. 24). The HN population displayed greater differences between lateral sides in five of eight investigated traits with ABW, APW, PPL, PPLF and PSL compared to AB. ABL, APL and FAP were more asymmetric in AB.

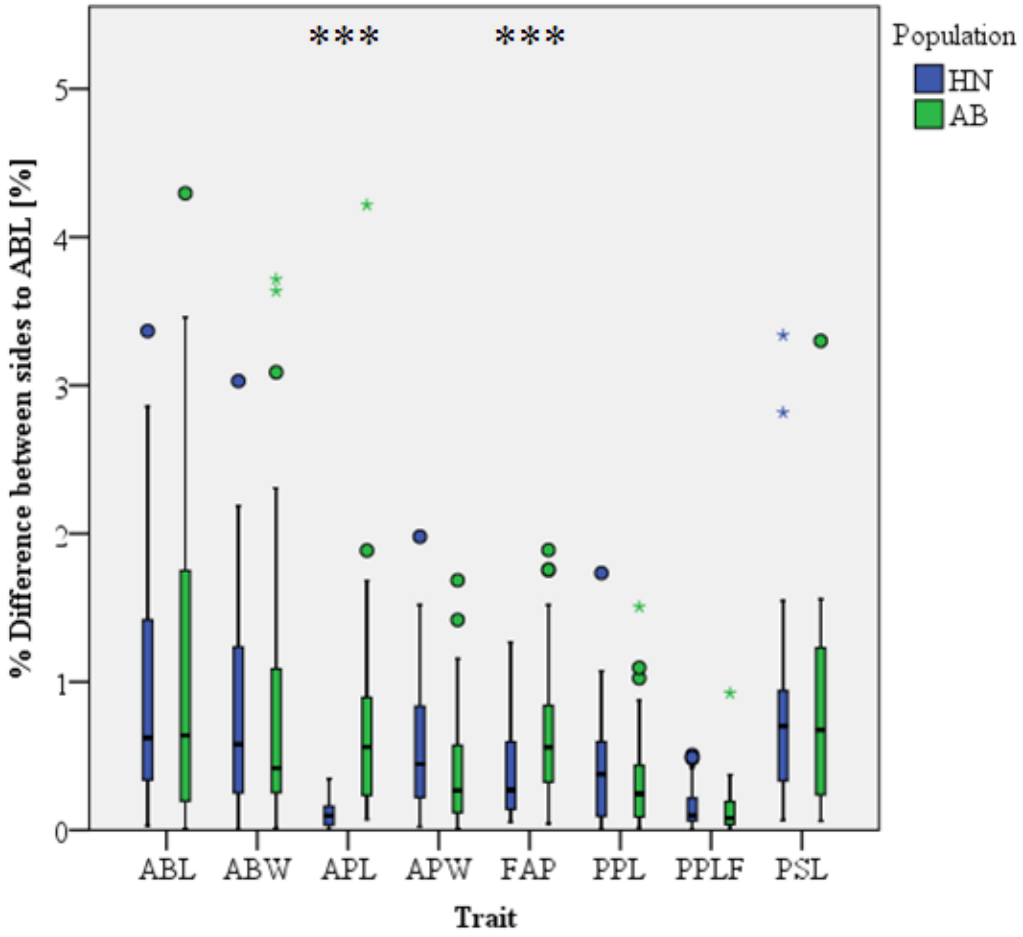

Figure 24: The difference between the left and the right side [%] to the distance ABL] of the HN population compared to the AB population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HN = Neubach; AB = Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.3.2.2.2. LK-AB

The LK compared to the AB population demonstrated significant values for APL ($p < 0.05$), FAP ($p < 0.05$) and PPLF ($p < 0.001$) (Fig. 25). APW, FAP, PPLF were longer on the left side, ABL and PSL were longer on the right side in both populations. APL had the same length on both sides in LK and was longer on the left side in AB. ABW was longer on the left side and PPL was longer on the right side in LK in contrast to AB.

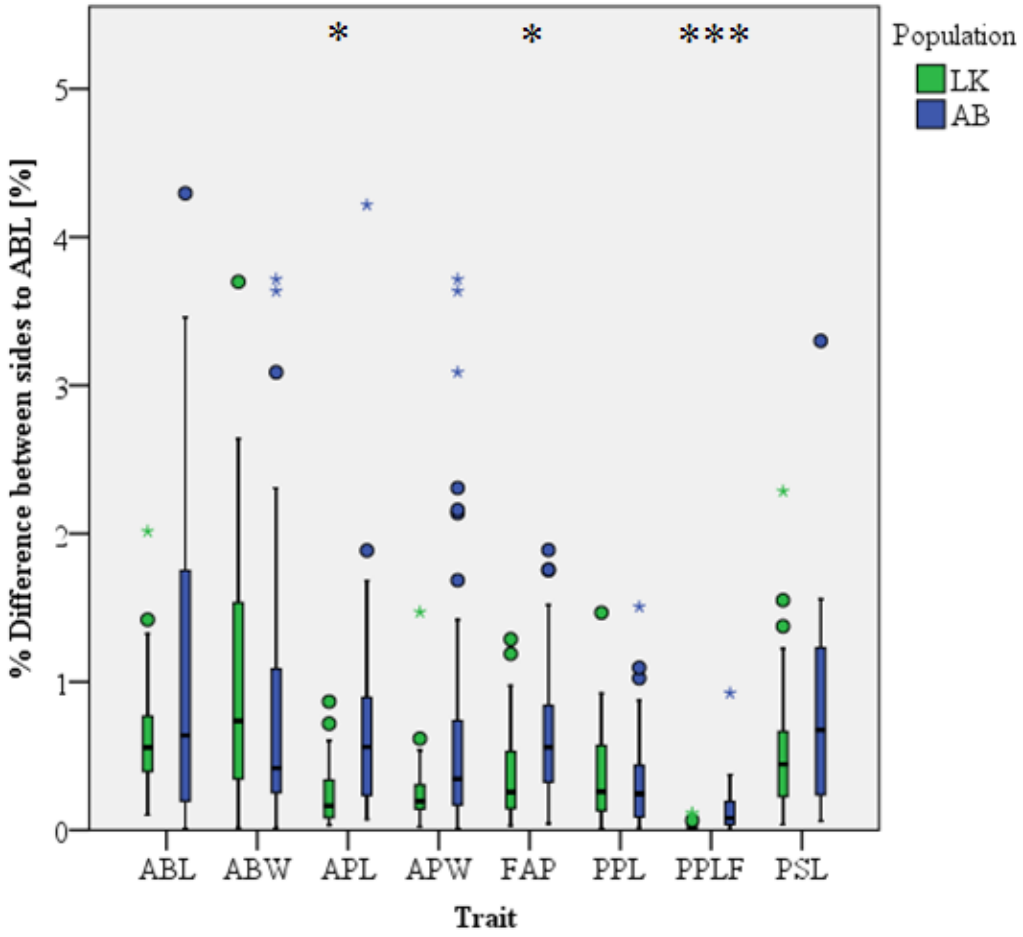

Figure 25: The difference between the left and the right side [%] to the distance ABL (ascending process length of the LK population compared to the AB population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). LK = Lustenauer Kanal; AB = Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The AB population demonstrated greater differences between the sides in six of eight investigated traits with ABL, APL, APW, FAP, PPLF and PSL. LK was more asymmetric in the two traits ABW and PPL.

3.3.2.2.3. LK-FUB

The LK population in comparison to FUB showed an almost significant value for APW ($p = 0.05$) (Fig. 26). APW and FAP were longer on the left side and ABL and PSL were longer on the right side in both populations. PPL was longer on the left in FUB in contrast to LK. APL did not differ in the length between the sides in LK and was longer on the left side in FUB. The trait PPLF displayed the same length on the left and right in FUB and was longer on the left side in LK. ABW was longer on the left side in LK in contrast to FUB.

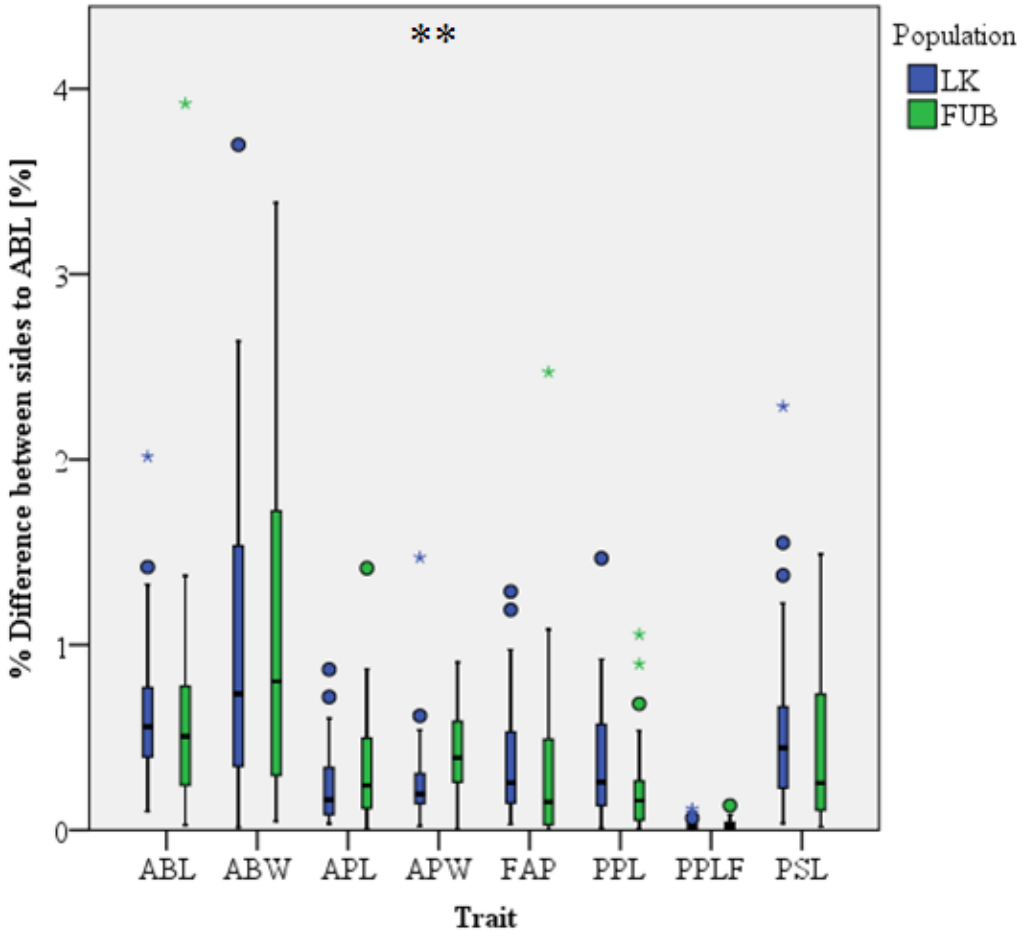

Figure 26: The difference between the left and the right side [%] to the distance ABL (ascending process length) of the LK population compared to the AB population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$). LK = Lustenauer Kanal; AB = Aiglbach. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The LK population showed greater differences in four of eight investigated traits with ABL, FAP, PPL and PSL. FUB population was more asymmetric in the four traits ABW, APL, APW and PPLF.

3.3.2.3. CO2

3.3.2.3.1. CO2 parents – CO2 control

The comparison of CO2 parents with CO2 control displayed a significant ABL value ($p < 0.05$) (Fig. 27). ABL was longer on the right side in both populations.ABW was longer on the left side and PSL was longer on the right side in CO2 control in contrast to CO2 parents.

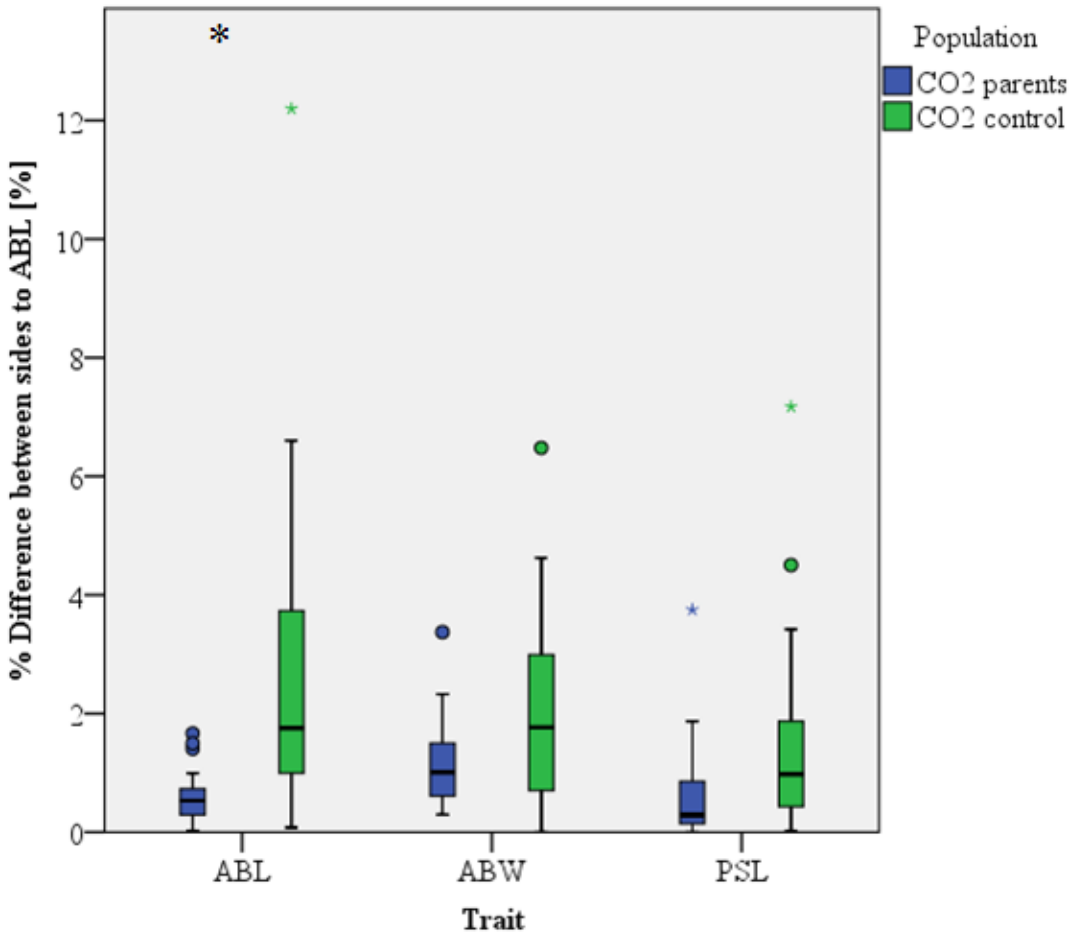

Figure 27: The difference between the left and the right side [%] to the distance ABL of the CO2 parents population compared to the CO2 control population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions. ABL = ascending process length, ABW = ascending process width, PSL = pelvic spine length.

The difference between lateral sides was higher in the CO2 control population in all three traits ABL, ABW and PSL compared to the CO2 parents.

3.3.2.3.2. CO2 parents – CO2 high

The CO2 parents population differed significantly from the CO2 high in the two traits ABL ($p < 0.05$) and ABW ($p < 0.01$) (Fig. 28). ABL was shorter on the left side in CO2 parents in contrast to CO2 high. ABW was longer on the left side in CO2 high and longer on the right side in the CO2 parents population. PSL was longer on the left side in CO2 parents in contrast to CO2 high.

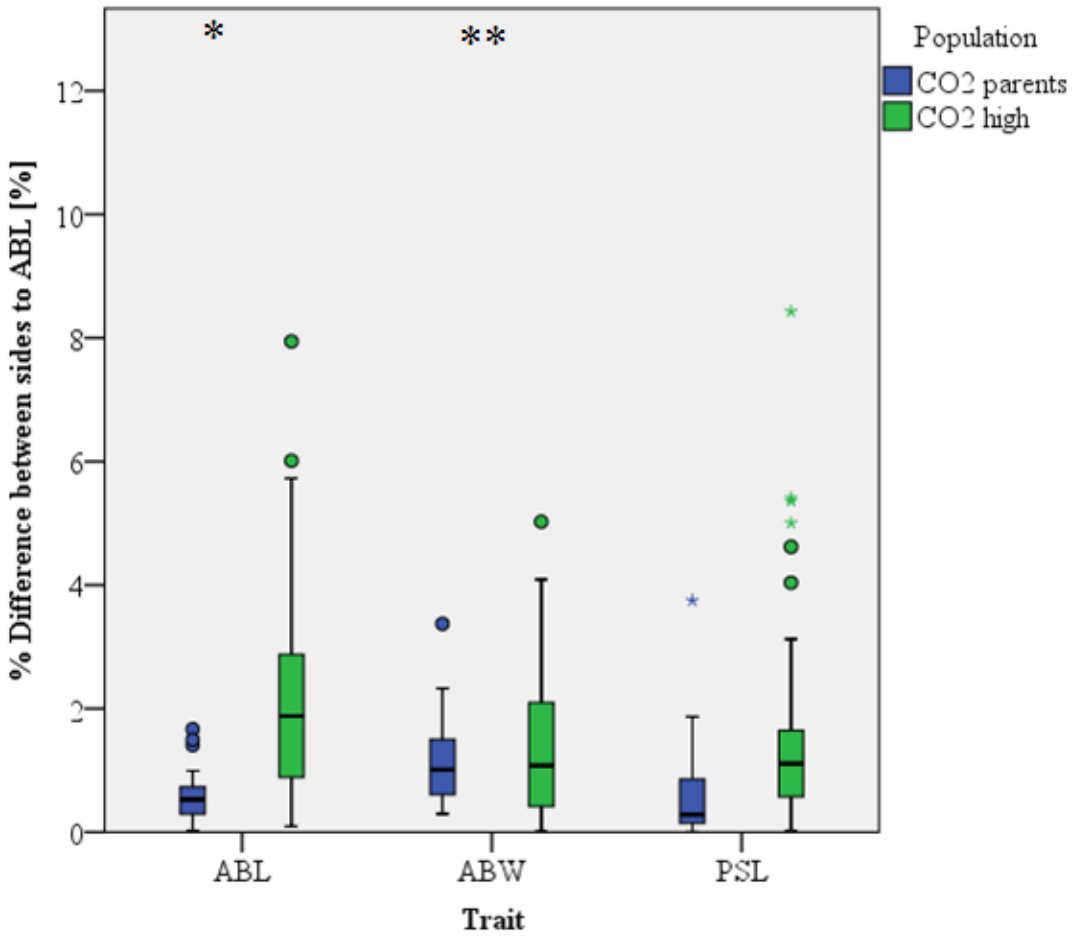

Figure 28: The difference between the left and the right side [%] to the distance ABL of the CO2 parents population compared to the CO2 high population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$). CO2 parents = marine adults from Sylt-Rømø Bight; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The difference between lateral sides was higher in the CO2 high population in all three traits ABL, ABW and PSL compared to the CO2 parents.

3.3.2.3.3. CO2 control – CO2 high

The CO2 control compared to the CO2 high population revealed no significant differences (Fig. 29). ABW was longer on the left side in both populations and PSL was longer on the right side. The trait ABL was longer on the left in CO2 high in contrast to CO2 control.

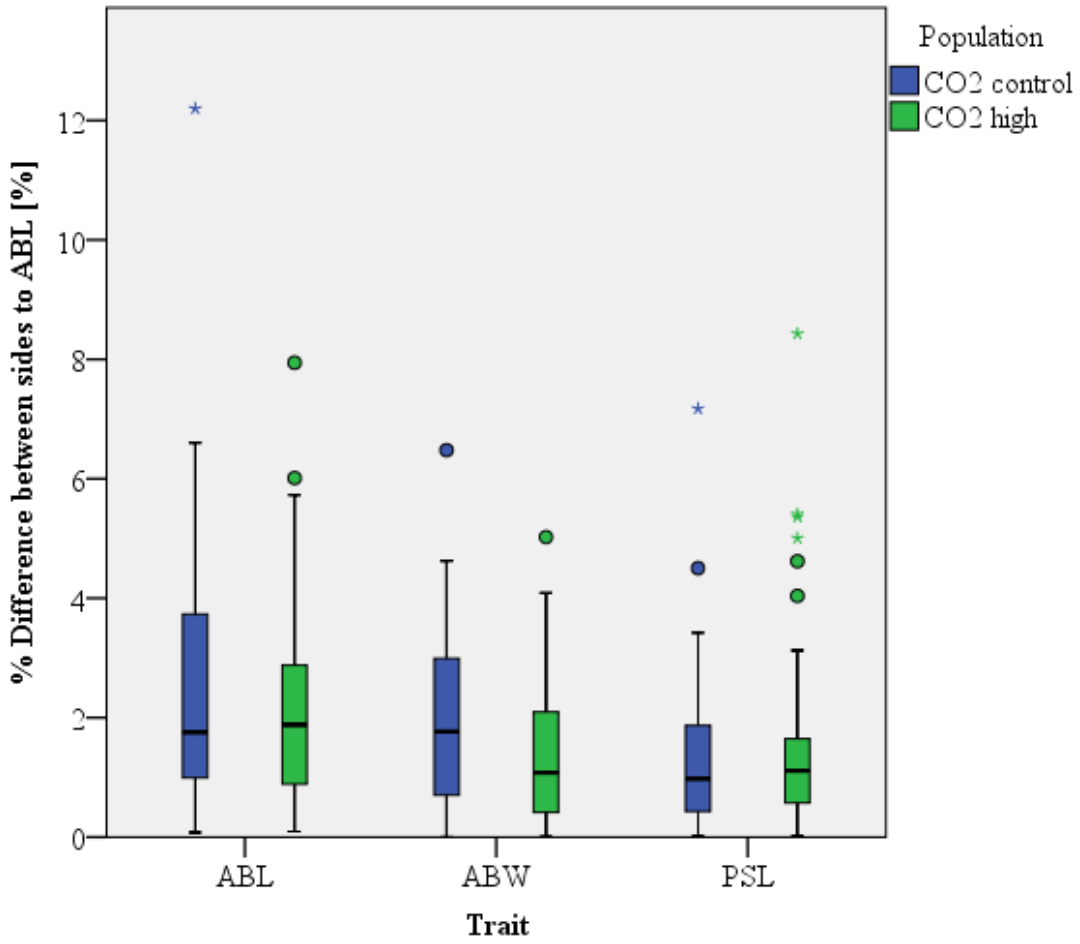

Figure 29: The difference between the left and the right side [%] to the distance ABL of the CO2 control population compared to the CO2 high population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under normal CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The CO2 control population revealed a smaller amount of differences between the sides in ABL and PSL and a higher amount of differences in ABW.

3.3.2.4. Temperature

3.3.2.4.1. T parents – T 17

PPLF in T parents had the same length on both sides and was longer on the left side in T 17. ABW and FAP were longer on the right side in T parents specimens. These traits were longer on the left side in T 17. APL and PSL were longer on the right side in T parents in contrast to T 17. ABL was longer on the right side and APW and PPL were longer on the left side in both populations.

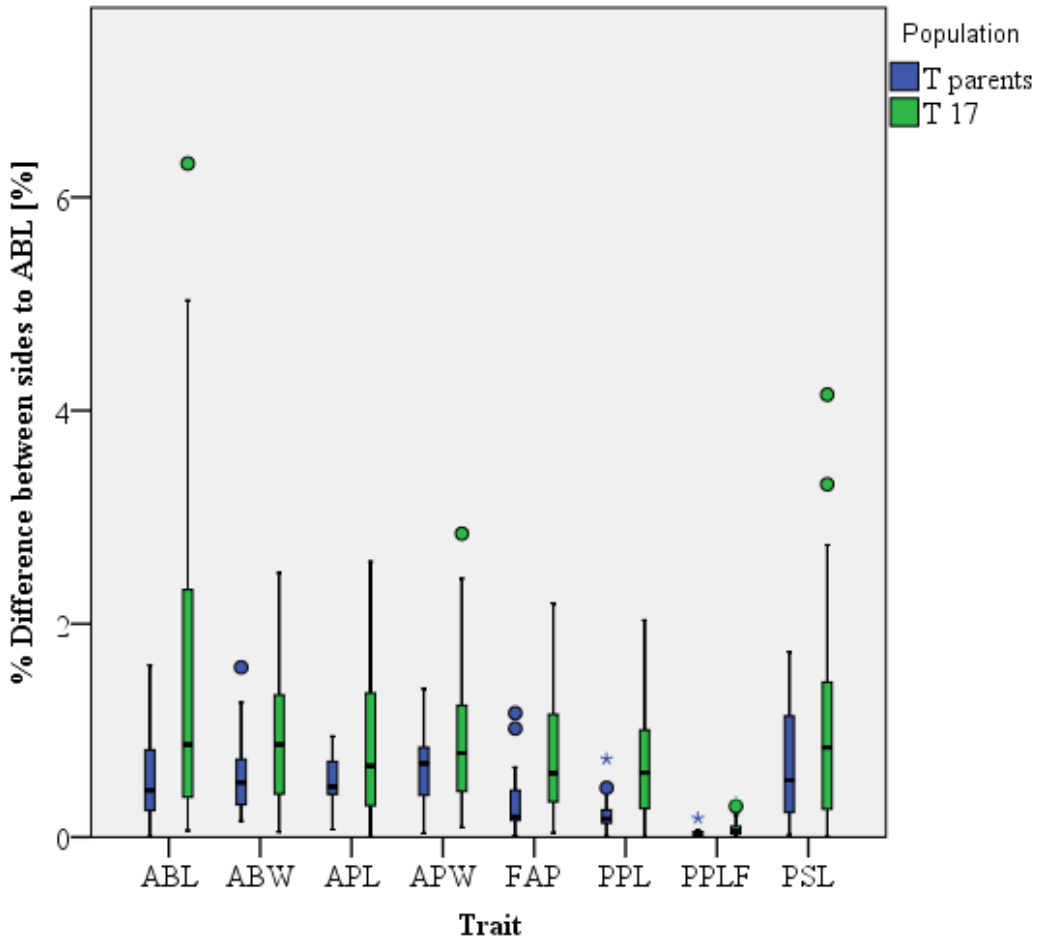

Figure 30: The difference between the left and the right side [%] to the distance ABL of the T parents population compared to the T 17 population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

The difference between the lateral sides was higher in the T 17 population in all eight investigated traits (Fig. 30).

3.3.2.4.2. T parents – T 21

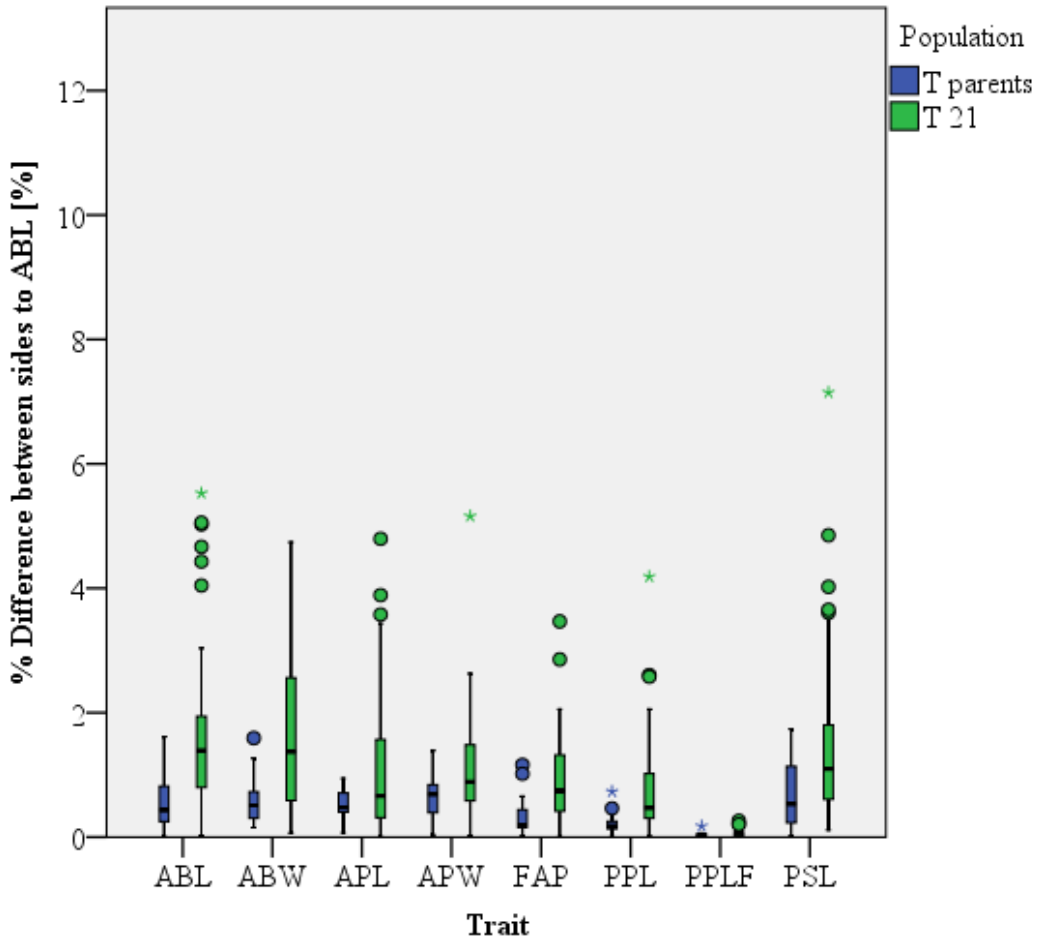

Figure 31: The difference between the left and the right side [%] to the distance ABL of the T parents population compared to the T 21 population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. T parents = marine adults from Sylt-Rømø Bight; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

T parents compared to T 21 demonstrated a highly significant value for PPLF ($p < 0.001$) (Fig. 31). The trait PPLF had the same length on the left and right side of the T parents individuals and was longer on the left side in T 21. Both populations had ABL and FAP longer on the right side. ABW, APL, PSL were longer on the left side in T 21 in contrast to T parents. The pelvic structures APW and PPL were longer on the left side in both populations. The difference between the lateral sides was higher in the T 21 population in all eight investigated traits compared to T parents.

3.3.2.3.3. T 17 – T 21

The comparison of T 17 and T 21 detected significant ABW ($p < 0.01$) and PSL ($p < 0.05$) values. The pelvic traits ABW, APL, APW, PPLF, PPL and PSL were longer on the left side whereas ABL was longer on the right in both populations. FAP was longer on the left side in T 17 in contrast to T 21.

T 21 showed more differences between the lateral sides in six of eight investigated traits with ABL, ABW, APW, FAP, PPLF and PSL (Fig. 32). APL and PPL were more asymmetric in T 17.

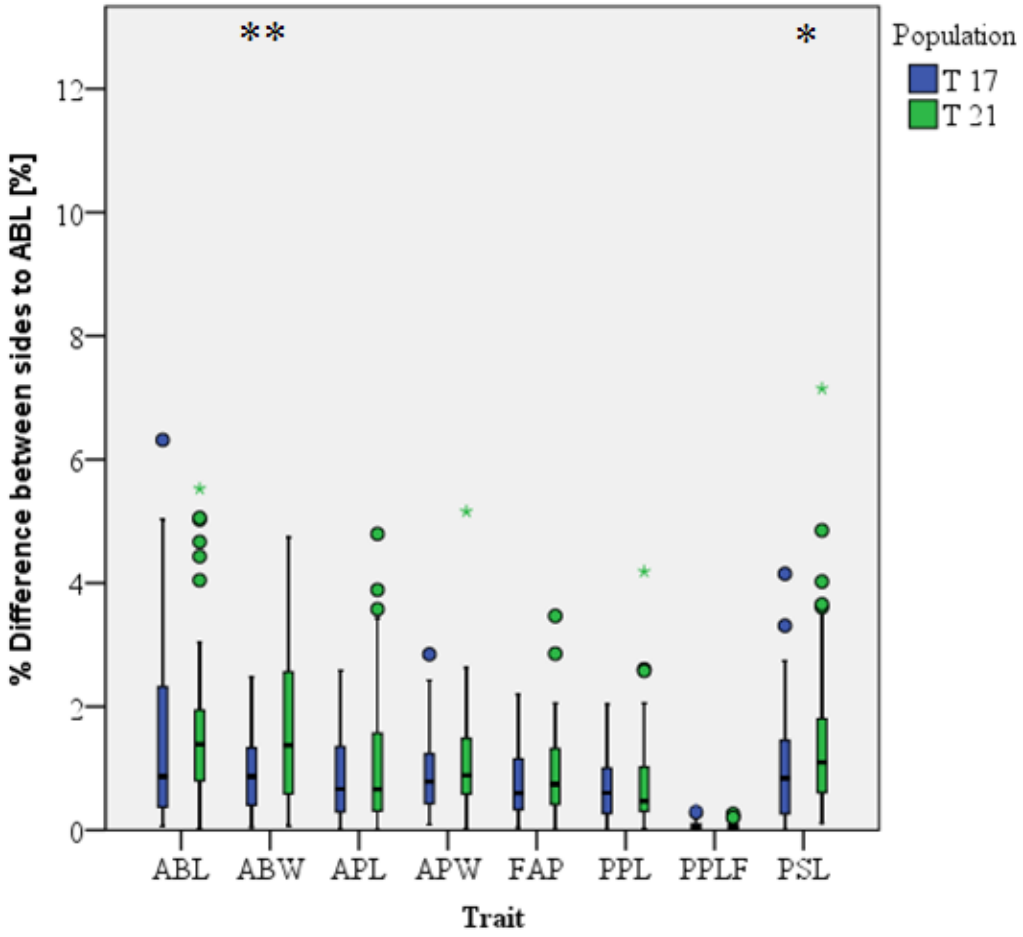

Figure 32: The difference between the left and the right side [%] to the distance ABL of the T 17 population compared to the T 21 population. Black lines mark the median. Circles mark single outliers. Stars mark extreme outliers. Asterisks demonstrate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABL = ascending process length, ABW = ascending process width, APL = anterior process length, APW = anterior process width, FAP = anterior process length from midline, PPL = posterior process length, PPLF = posterior process to midline of anterior processes, PSL = pelvic spine length.

3.4. Dorsal edge of the ascending processes

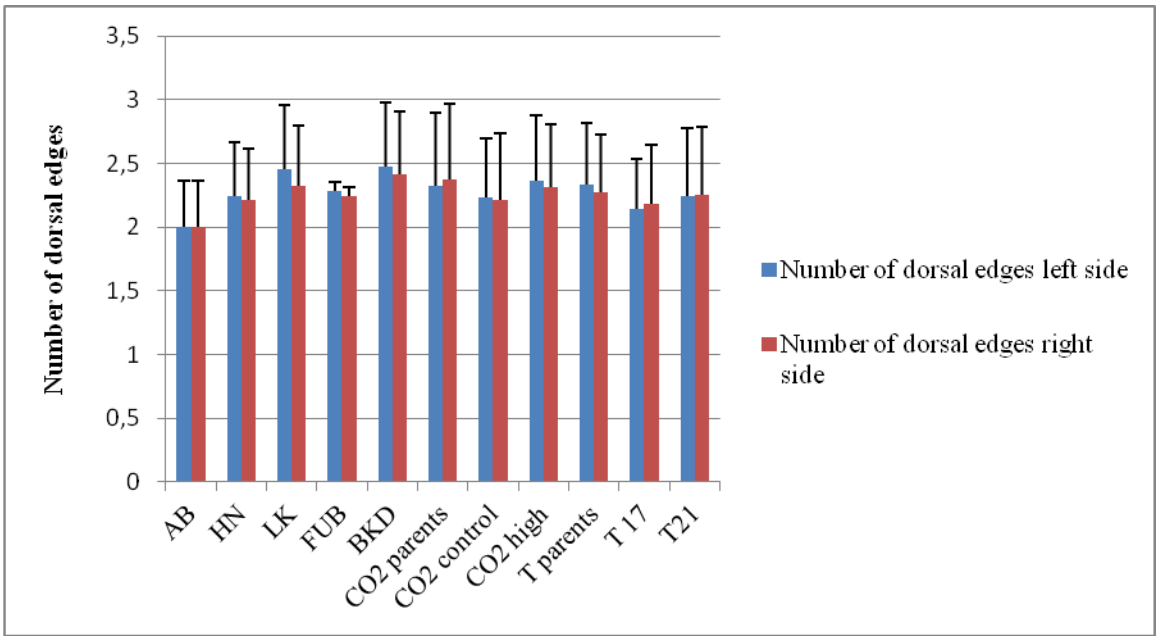

Figure 33: Mean number and Standard deviation (SD) of dorsal edges on left and right side of the ascending process. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Table 12: Mean number of dorsal edges of the ascending process and Standard deviation (SD). AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Population	**n dorsal edges left**	**n dorsal edges right**	**SD left**	**SD right**
AB	2.00	2.00	0.36	0.36
HN	2.24	2.21	0.43	0.41
LK	2.45	2.32	0.51	0.48
FUB	2.28	2.25	0.07	0.07
BKD	2.47	2.41	0.51	0.50
Eltern CO2	2.32	2.37	0.58	0.60
CO2 control	2.23	2.21	0.47	0.53
CO2 high	2.36	2.31	0.52	0.50
Eltern Temperatur	2.33	2.27	0.49	0.46
TF 17	2.14	2.18	0.40	0.47
TF 21	2.24	2.25	0.54	0.54

The number of dorsal edges of the ascending process ranged from one to three (Fig. 33). The population from AB had the smallest number of edges and the mean of the left and the right side was the same (Table 12). All the other populations had asymmetric numbers of dorsal edges of the ascending process. Some specimens displayed a different number of dorsal edges between their lateral sides (Table 13).That was referred to as asymmetry in this study.

The ascending branch showed more dorsal edges on the left side than on the right in CO2 high, LK, BKD, HN, FUB, T parents. A higher number of edges on the right than on the left side could be observed in T 17, T 21 and CO2 parents.

Table 13: The number of symmetric and asymmetric specimens concerning the dorsal edges of the ascending process in total and in percent. n = Number of specimens. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C.

Population	**n**	**n symmetric**	**n asymmetric**	**% symmetric**	**% asymmetric**
AB	34	34	0	100.00	0.00
HN	34	33	1	97.06	2.94
LK	33	23	10	69.70	30.30
FUB	33	24	9	72.73	27.27
BKD	34	32	2	94.12	5.88
CO2 parents	19	16	3	84.21	15.79
CO2 control	56	47	9	83.93	16.07
CO2 high	59	50	9	84.75	15.25
T parents	15	14	1	93.33	6.67
T 17	56	50	6	89.29	10.71
T 21	59	52	7	88.14	11.86

The AB population revealed the smallest amount of asymmetry concerning the edges of the ascending process and LK displayed the highest amount of asymmetric specimens.

The number of one dorsal edge of the ascending process was quite rare and only common in populations of AB, CO2 parents, CO2 fish (CO2 control and CO2 high) and T fish (T 17 and T 21). The pelvic traits ABL, ABW were smallest and narrower in specimens with one dorsal edge (Tab. 14).

Table 14: Mean of the lengths ABLL [mm], ABLR [mm], ABWL [mm], ABWR [mm] in specimens with different numbers of dorsal edges. AB = Aiglbach; HN = Neubach; LK = Lustenauer Kanal; FUB = Fussacher Bay; BKD = Brede Å system, Kisbaek creek; CO2 parents = marine adults from Sylt-Rømø Bight; CO2 control = offspring of marine adults from Sylt-Rømø Bight reared under ambient CO2 conditions; CO2 high = offspring of marine adults from Sylt-Rømø Bight reared under elevated

CO2 conditions; T parents = marine adults from Sylt-Rømø Bight; T 17 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 17 ° C; T 21 = offspring of marine adults from Sylt-Rømø Bight reared under a temperature of 21 ° C. ABLL = ascending process length, left side; ABLR = ascending process length, right side; ABWL = ascending process width, left side; ABWR = ascending process width, right side. 1 – 3 = number of dorsal edges.

Population	**ABLL (1)**	**ABLR (1)**	**ABWL (1)**	**ABWR (1)**	**ABLL (2)**	**ABLR (2)**	**ABWL (2)**	**ABWR (2)**	**ABLL (3)**	**ABLR (3)**	**ABWL (3)**	**ABWR (3)**
AB	5.75	5.73	1.50	2.04	6.23	6.17	2.56	2.64	6.22	5.92	2.98	2.86
HN	-	-	-	-	5.18	5.30	2.19	2.17	5.19	5.21	2.36	2.40
LK	-	-	-	-	7.68	7.70	3.20	3.08	7.66	7.54	3.66	3.17
FUB	-	-	-	-	8.42	8.69	3.46	3.76	8.74	8.83	4.06	4.41
BKD	-	-	-	-	6.80	6.94	2.67	2.93	7.02	7.08	3.38	3.43
CO2 parents	4.71	4.89	2.06	1.78	6.53	6.49	2.75	2.94	6.63	6.84	3.19	3.22
CO2 fish	2.98	2.70	1.10	1.03	3.19	3.10	1.20	1.20	3.18	3.23	1.36	1.36
T parents	-	-	-	-	6.97	7.06	3.19	3.13	7.25	7.67	3.64	3.92
T fish	3.36	3.34	1.23	1.23	3.75	3.75	1.50	1.42	3.54	3.81	1.70	1.72

The freshwater populations of AB and HN revealed the smallest values of the length and width of the ascending process, while FUB showed the largest ones. The largest adult oceanic population concerning ascending process traits (ABLL, ABLR, ABWL, ABWR) was BKD, the smallest was CO2 parents.

The CO2 and temperature fish demonstrated the smallest lengths of these traits. The CO2 fish displayed the smallest sizes of all investigated populations.

4. Discussion

4.1. Size (SL)

Generally sticklebacks from large water bodies (the sea or lakes) are larger in size than those inhabiting streams and brooks (Moser et al., 2012). This is similar to that what has been found in this study: the anadromous sticklebacks and the sticklebacks from Lake Constance were larger than those from any investigated stream population (Fig. 8; Table 4). It has been shown that larger body size may increase the body diameter (Reimchen, 2000) and, together with longer spines this may aid against predatory pressure by gape-limited predators. But this is not necessarily related to the length but to the height of the specimens (Reimchen, 1983, 2000; Bergstrom and Reimchen, 2000; Reimchen and Nosil, 2002; Klepaker et al., 2012). Nevertheless the effectiveness of the defensive complex does not necessarily depend on a large body size. It could also be influenced through the length of dorsal spines as mentioned above or the number of bony lateral plates (Reimchen, 1983, 2000; Bergstrom and Reimchen, 2000). A different situation occurred in the juvenile fish (temperature and CO2 treatment). The larger SL of the T juveniles compared to the CO2 juveniles was likely due to the different ages of these fish. The T fish were older and therefore larger than the CO2 juveniles. Temperature fish (the same as Ramler et al. 2014) were larger at a temperature of 17 °C , the mean surface temperature of the North Sea in summer, than the fish reared at a temperature of 21 °C indicating that developmental conditions were most stable at this temperature compared to the increased temperature condition (Ramler et al., 2014). The population raised at elevated CO2 was larger than the CO2 control group which suggests that the growth rate of juvenile sticklebacks was not negatively affected by the elevated CO2 level. This result was inconsistent with the study of Pimentel et al. (2014) where a negative effect of growth in fish larvae was observed. The SL exhibited a sexual dimorphism in size (Fig. 9). Females were generally longer than males in the three populations with determined sex. Two of the populations (BKD and FUB) showed a significant difference between sexes while the T parents did not. But this result was likely biased by the low number of specimens (n = 15) and because the number of females was twice as much as the number of males in the T parents population. This result was consistent with other studies (Kitano et al., 2007, 2012).

4.2. Pelvic traits

Generally the pelvic complex is well developed in the threespine stickleback as it is part of the defensive complex, a set of bony elements protecting the fish against predatory pressure. Nevertheless it may be advantageous to reduce it by reaching a higher growth rate, better swimming abilities, decreased drag and a higher agility if predatory pressure is low or reduced (Bergstrom and Reimchen, 2003; Klepaker et al., 2012). Pelvic elements are lost in a characteristic order: (1) pelvic spine, (2) posterior process, (3) ascending process, (4) anterior process (Blouw and Boyd, 1992; Bell and Foster, 1994; Bell et al., 2007). The pelvis can also be totally reduced and this was observed in three genera of Gasterostoidae (*Culaea, Gasterosteus, Puntigitius*) (Nelson, 1971; Blouw and Boyd, 1992; Bell and

Foster, 1994; Ziuganov and Zotin, 1995; Cresko et al., 2004; Shapiro et al., 2004; Bell et al., 2007; Klepaker et al., 2013). Pelvic reduction is likely due to a genetic basis and it is suggested that one major and four minor chromosome regions are involved (Shapiro et al., 2004; Peichel, 2005; Wootton, 2009). A pelvic girdle left-biased directionality was observed in many studies (Nelson, 1971; Reimchen, 1997; Shapiro et al., 2006; Coyle et al., 2007; Reimchen and Bergstrom, 2009) and can also appear together with a general pelvic reduction (Mazzi and Bakker, 2001; Bell et al., 2007). That may be caused by Pitx2, a homeobox-containing transcription factor, which is only expressed on the left side (Shapiro et al., 2004, 2006; Peichel, 2005; Bell et al., 2007; Coyle et al., 2007; Cresko et al., 2007). A general left bias of pelvic spines could not be observed in this study although some populations exhibited larger spines on the left side and the kind of asymmetries were not determined.
Many taxa show a negative correlation between fluctuating asymmetry and constituents of fitness (Bergstrom and Reimchen, 2000, 2003; Reimchen and Nosil, 2001a; Mazzi et al., 2004; Reimchen and Bergstrom, 2009). Fluctuating asymmetry can reflect relative fitness and is associated with reduced immunocompetence, increased susceptibility to parasitism (Reimchen, 1997, 2002; Reimchen and Nosil, 2001a; Loehr et al., 2013), reduced survivorship and avoidance by potential mates (Bergstrom and Reimchen, 2002; Reimchen and Bergstrom, 2009). Studies showed that stickleback specimens with a asymmetric pelvis are more predisposed for parasitic infections than symmetric individuals that could be due to a reduced immunocompetence in asymmetric fish (Reimchen, 1997; Reimchen and Nosil, 2001b). Asymmetries are disadvantages especially in males of threespine sticklebacks, because female choose their mating partners based on their asymmetries and especially the symmetry of the pelvic spines seems to be of high importance (Blouw and Boyd, 1992; Paepke, 2002; Mazzi et al., 2004; Bakker et al., 2006).
The pelvic trait PPLF showed a low degree of asymmetry in all populations, indicating that this trait is very conservative (Table 9). All the other traits were quite variable. The differences between the sides were quite small in all populations.

4.3. Left-right side differences

All populations showed no distinct differences in the left/right symmetry in all investigated pelvic traits (Table 8; Table 9). This indicates that all these structures are important for predatory defence. These traits may all be very conservative because even the populations under a low to very low predatory pressure, which were expected to show higher asymmetries, did not differ significantly. The BKD and T 17 populations were exceptions with each one of them demonstrating a significant to highly significant width of the anterior processus (APW).

4.4. Inter-population comparison

The inter-population comparison revealed some significant to highly significant differences regardless of predatory pressure, habitat, CO_2 or temperature.

4.4.1. Natural selection

Predatory pressure

A gradient was found concerning morphological traits and predatory pressure. There were less significant differences between the HN and AB population that were both under a low and very low predatory pressure, whereas the FUB population under a high predatory pressure differed greater from the other populations (Fig. 22; Fig. 23; Fig. 24). Predatory pressure might be the most important factor for morphological changes. In general structures that are necessary for the survival of a prey species should be under a strong selective pressure in habitats with a high predatory pressure (Bergstrom and Reimchen, 2003).

The FUB population, under a high predatory pressure, demonstrated higher symmetries in the pelvic traits compared to AB and the HN populations, both under very low to low predatory pressure. These differences along a predatory gradient are in accordance with the results of Bergstrom and Reimchen (2003). Nevertheless FUB revealed more asymmetries in ABW and APW compared to AB and in ABW and APL compared to HN. ABW is probably already too much reduced in width in both populations compared to FUB and the ascending process is much wider and also longer in FUB which could explain a higher fluctuation in these two measured traits. Comparison of the two populations under low (HN) and very low (AB) predatory pressure revealed that HN was more asymmetric in most of the traits. The specimens of AB did not show a higher amount of asymmetries than HN, but the opposite result.

The reason could be that it probably does not matter how much the predatory pressure is reduced as long as it is low. The advantages of symmetric traits may be too small and their development may be too costly in habitats with a low predatory abundance (Bergstrom and Reimchen, 2002). Some studies even found advantages in asymmetry (discussed in Bergstrom and Reimchen, 2000).

Previous studies found differences in shape between habitats in stickleback populations (e.g. Bergstrom and Reimchen, 2002; Spoljaric and Reimchen, 2007; McGuigan et al., 2010; Aguirre and Bell, 2012). These differences are interpreted as an adaptation to different habitats. Significant differences could be found between the stream-stream as well as between stream-lake populations. Contrary to that the result of this study showed less significant differences in the comparison between the stream-lake population than between stream-stream populations (Fig. 24; Fig. 25; Fig. 26). These results are likely not due to habitat differences but because of the differences in the predatory pressure.

4.4.2. Anthropogenic stressors

Abiotic stressors like temperature and pH-value may be very important in the future as mean water temperatures and the number of extreme environmental events are predicted to increase due to climate change (Dittmar et al., 2014; Metzger et al., 2016; Schade et al., 2016). Temperatures models predicted an increase of temperature by 3-4 °C due to global warming (Moran et al., 2010; Ramler et al.,

2014; Pimentel et al., 2016; Shama et al., 2016). A higher temperature may make specimens more vulnerable to other stressor (for example acidification or parasitism or diseases) (Pimentel, Faleiro, et al., 2014; Pimentel et al., 2016). Additionally a decrease in the pH value is expected ("acidification of the oceans") (Schade et al., 2014 b; Ahnelt et al., 2016; Schade et al., 2016)

Especially early life stages such as eggs and embryos of aquatic organisms are going to be confronted with the challenging conditions (decreasing pH; increasing temperatures) of the changing climate (Moran et al., 2010; Pimentel et al., 2014 a; Pimentel et al., 2014 b; Schade et al., 2014 a; Pimentel et al., 2015, 2016; Shama et al., 2016). The average sea surface temperature and acidities already greatly deviate from the values that were existent in the sea for up to millions of years (Shama et al., 2016).

The CO2 parents were less asymmetric in all of the investigated traits (Fig. 27; Fig 28). These results are likely caused by the low number of specimens (n = 17).

The comparison of CO2 high and CO2 control specimens revealed no significant differences in any of the investigated traits. Nevertheless a somewhat higher asymmetry was in CO2 high in ABL and ABW (Fig. 29). Some studies observed a left bias in pelvic spine length affected by an elevated CO2 level (Nelson, 1971; Reimchen, 1997; Shapiro et al., 2006; Coyle et al., 2007; Reimchen and Bergstrom, 2009). Mazzi and Bakker (2001) found significant asymmetries in the pelvic spine length in sticklebacks reared under elevated CO2. Such significant differences were not found in this study (Fig. 29).

Bergstrom and Reimchen (2002) analysed sticklebacks from localities that varied in environmental conditions and discovered that populations from lowland (characterized by very shallow, acidic and dystrophic lakes) showed the highest composite fluctuating asymmetry index, compared to the mountains that showed an increase in water colour and pH. Another study of Berstrom and Reimchen also showed that specimens were most asymmetric in acidic habitats and also possessed only a small number of lateral plates, but these results were probably due to a lower visibility to predators in turbid, acidic habitats rather than to abiotic factors (Bergstrom and Reimchen, 2003).

The T parents population was less asymmetric in all pelvic traits compared to T 17 and T 21 (Fig. 30; Fig. 31). Like in the CO2 parents the results are likely caused by the low number of T parents (n = 15).The specimens reared under elevated temperature (T 21) were more asymmetric in most of the traits (Fig. 32) but differed significantly only in the width of the ascending branch and in the pectoral spine length. This indicates that the development was quite unstable because it was accelerated at the temperature of 21 °C (Ramler et al., 2014). Asymmetries in these two traits, especially in the spine length may affect not only predator avoidance but also sexual selection. Females chose males also by the symmetry of their pelvic spines (Mazzi et al., 2003).

Conclusion

The SL of the investigated stickleback populations revealed a sexual dimorphism that was also discovered in other studies. Slight differences concerning the pelvic traits could be found between the left and right side of threespine sticklebacks in all of the observed populations. But these differences were quite small indicating that these traits are very conservative. The population under predatory pressure was more asymmetric in most of the pelvic traits compared to the populations under a low predatory pressure. Many previous studies revealed that sticklebacks are more symmetric under high predatory pressure in many different traits. Contrary to this, differences in the habitat (lake-stream) seemed to have no effect on the development of the pelvis. Although the CO2 high group revealed more asymmetries than the CO2 control no significant differences could be observed. Contrary to Mazzi and Bakker (2001) a general left side bias could not be detected in this study. Temperature had a higher effect onto the development in structures as most traits were more asymmetric in T 21 and two traits were significantly asymmetric influencing predatory avoidance and sexual selection. Possibly the elevated temperature accelerated the development and caused a higher degree of asymmetries.
To sum all the results up natural selection such as a low predatory pressure as well as anthropogenic stressors as elevated temperature and CO2 level caused no very distinct changes in the symmetry of pelvic elements in the investigated threespine sticklebacks. Sticklebacks revealed the ability to cope with a wide range of different stressors like in many other previous studies.

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