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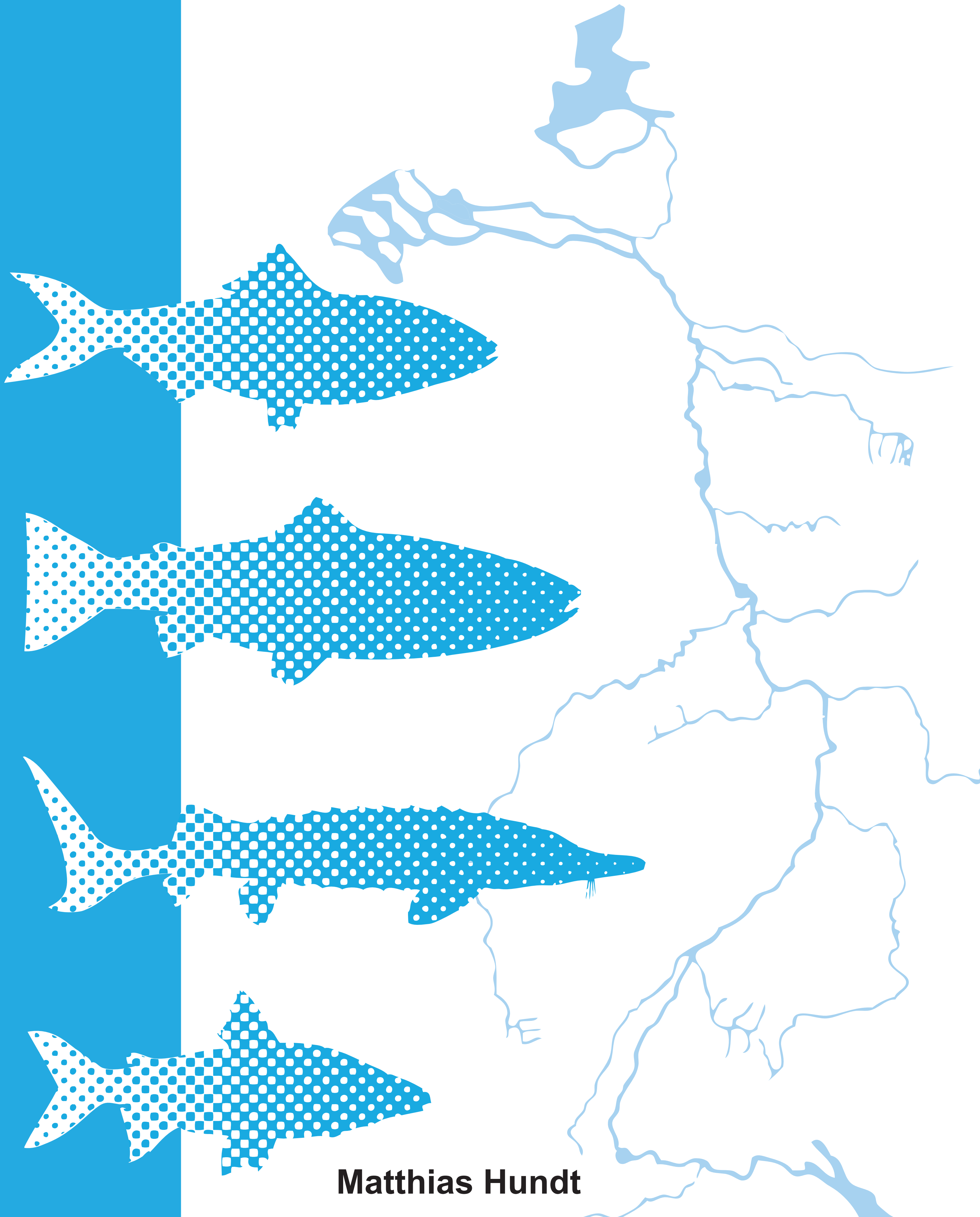

Matthias Hundt

Aquaculture, conservation and restoration of anadromous fish populations of River Rhine with particular regard to the re-introduction of the Allis shad *Alosa alosa*.

by Matthias Hundt

from Lichtenfels

Accepted Dissertation thesis for the partial fulfillment of the requirements for a Doctor of Natural Sciences Fachbereich 7: Natur- und Umweltwissenschaften

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Abbreviations & Explanations

ANOVA: Analysis of variance
APE: Average precision index
ARA: Arachidonic acid
BMU: Federal ministry for the environment, nature conservation and nuclear safety (*In German:* Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit)
CEMAGREF: National center for agricultural mechanization, rural engineering, water and forests (*In French:* Centre National du Machinisme Agricole du Génie Rural des Eaux et des Forêts)
CPE: Cytopathic effect
DHA: Docosahexaenoic acid
dph: Days post hatch
dpi: Days post infection
EPA: Eicosapentaenoic acid
FA: Fatty acid
FAME: Fatty acid methyl esther
GLM: Generalized linear modelling
GSI: Gonad somatic index
HPLC: High performance liquid chromatography
ICPR: International commission for the protection of the Rhine
IRSTEA: National research institute of science and technology for environment and agriculture (*In French:* Institut national de recherche en sciences et technologies pour l'environnement et l'agriculture)
IUCN: International union for the conservation of nature
MD: Microdiet
MIGADO: Association for the restoration and management of migratory fish in the Garonne-Dordogne basin (in French: Association pour la restauration et la gestion des poisons Migrateurs du basin de la Garonne et de la Dordogne)
MUFA: Monounsaturated fatty acid
OTC: Oxytetracycline
PAH: Polycyclic aromatic hydrocarbons
PCB: Polychlorinated biphenyl
PCR: Polymerase chain reaction
PUFA: Polyunsaturated fatty acid
RAP: Rhine action programme
RAS: Recirculating aquaculture system
SAFA: Saturated fatty acid
TC: Tetracycline
WFD: Water framework directive
YOY: Young of the year (fish at their first year of life)

Chapter I: General Introduction

Decline of native anadromous fish in Germany

Diadromous fish species as defined by Myers (1949) are fish migrating between the sea and freshwater in order to achieve their lifecycle. Catadromous fish species spend the major part of their lifecycle in freshwater and move to sea in order to reproduce, while anadromous fish spend most of their life in the sea and migrate to freshwater to breed.

In Germany the presence of 19 indigenous diadromous fish species has been historically recorded (Table I.1). Of these 19 species 16 perform anadromous migration, while 3 species are catadromous. The largest group of anadromous fish species in Germany belongs to the family Acipenseridae (Table I.1). Three species of sturgeon are known from the Danube River and its tributaries: the Beluga sturgeon *Huso huso* (L., 1758), the Sterlet *Acipenser ruthenus* (L., 1758) and also the Russian sturgeon *Acipenser gueldenstaedtii* (Hensel & Holčík 1997; Waldman 2000). Until recently the North Sea basin and the Baltic Sea basin were believed both to be inhabited by the European sturgeon *Acipenser sturio* only, however genetical evidence suggests that the Baltic area was colonized by the Atlantic sturgeon *Acipenser oxyrinchus* (Mitchill, 1815) in the middle ages and replaced the native European sturgeon in that area. The families Petromyzontidae, Clupeidae, Salmonidae and Coregonidae were in Germany represented with 2 anadromous species respectively (Table I.1). The most renown species of these families is probably the Atlantic salmon *Salmo salar* (L., 1758), which is considered an important flagship species and represented one of the most important riverine fishing resources of the 19th century (De Groot 2002; Ingendahl & Beeck 2011). Also the North Sea houting *Coregonus oxyrhinchus* (L., 1758), an anadromous coregonid species, as well as the Allis shad *Alosa alosa* (L., 1758) have to be regarded as important commercial fish species of the 19th century. Although three species in Germany are principally defined as catadromous (Table I.1) only the European eel *Anguilla anguilla* (L., 1758) can be considered an obligatory catadromous species spending extensive periods within freshwater habitats (Ibbotson *et al.* 2002; Maes, Stevens & Breine 2007).

According to Jonsson, Waples & Friedland (1999) diadromous fish face a relatively higher risk of extinction or population decline than obligate freshwater or marine species. Of the circa 230-250 diadromous species worldwide around 18% are considered to be of any conservation concern, whereas only 5% of all fish species as a whole are considered endangered, threatened, vulnerable, rare or of indeterminate status. The relatively higher risk of population decline or even extinction can be mainly explained by the obligate migratory nature of these fish, which in consequence mostly face a large array of threats, which often work in combination (McDowall 1992; Jonsson, Waples & Friedland 1999).

Table I.1 Summary of indigenous diadromous fish species in Germany: EX: globally extinct, RE: regionally extinct, CR: critically endangered, VU: vulnerable, NT: near threatened, LC: least concern, NE: not evaluated. Information based on: Lelek & Köhler 1990; Hensel & Holčík 1997; Waldman 2000; Ludwig *et al.* 2008; Lassalle & Rochard 2009. Global conservation status (IUCN Global) was derived from the IUCN red list of species (http://www.iucnredlist.org/). European conservation (IUCN Europe) status according to Freyhof and Brooks (2011). German conservation (RL Ger) status according to Freyhof (2009). Criteria of the German red list system were transcribed to IUCN criteria as proposed by Ludwig *et al.* (2006).

Species	Family	Common name	Type	IUCN Global	IUCN Europe	RL Ger
Lampetra fluviatilis (L., 1758)	Petromyzontidae	River lamprey	A	LC	LC	VU
Petromyzon marinus (L., 1758)	Petromyzontidae	Marine lamprey	A	LC	LC	NT
Acipenser gueldenstaedtii (Brandt and Ratzeburg,	Acipenseridae	Russian sturgeon	A	CR	CR	RE
Acipenser stellatus (Pallas, 1871)	Acipenseridae	Stellate sturgeon	A	CR	CR	RE
Acipenser oxyrinchus (Mitchell, 1815)	Acipenseridae	Baltic sturgeon	A	NT	NE	RE
Acipenser sturio (L., 1758)	Acipenseridae	European sturgeon	A	CR	CR	RE
Huso huso (L., 1758)	Acipenseridae	Beluga sturgeon	A	CR	CR	RE
Anguilla anguilla (L., 1758)	Anguilidae	European eel	C	CR	CR	NE
Alosa alosa (L., 1758)	Clupeidae	Allis shad	A	LC	LC	CR
Alosa fallax (Lacepede, 1803)	Clupeidae	Twaite shad	A	LC	LC	NE
Vimba vimba (L., 1758)	Cyprinidae	Vimba	A	LC	LC	VU
Osmerus eperlanus (L., 1758)	Osmeridae	Smelt	A	LC	LC	NT
Coregonus maraena (Bloch 1997)	Coregonidae	Maraena whitefish	A	VU	VU	VU
Coregonus oxyrinchus (L., 1758)	Coregonidae	North Sea houting	A	EX	EX	EX
Salmo salar (L., 1758)	Salmonidae	Atlantic salmon	A	LC	NE	CR
Salmo trutta (L., 1758)	Salmonidae	Brown trout	A	LC	LC	LC
Liza ramada (Risso, 1826)	Mugilidae	Thin-lipped mullet	C	LC	LC	NE
Gasterosteus aculeatus (L., 1758)	Gasterosteidae	Three-spined Stickleback	A	LC	LC	LC
Platichthys flesus (L., 1758)	Pleuronectidae	European flunder	C	LC	LC	NE

In Germany at least 15 out of 19 (79%) indigenous diadromous species are under some kind of conservation concern (Table I.1). Of all anadromous species in Germany only populations of the Three-spined stickleback *Gasterosteus aculeatus (L., 1758)* can currently be regarded as above any risk category. All five sturgeon species are regionally extinct in Germany (Table I.1) and the European sturgeon *A. sturio* is currently only known to reproduce in one single remaining river system (Gironde-Garonne-Dordogne System, France) and immediately threatened by worldwide extinction (Gessner *et al.* 2011). The North Sea houting *C. oxyrinchus* is classified to be globally extinct since the 1940ies by the IUCN (Freyhof & Schoter 2005; Freyhof & Brooks 2012) (Table I.1), however other authors claim that this species is identical with *C. maraena*, which can currently be found in the North and Baltic Sea (Dierking *et al.* 2014) and genetic evidence supporting a separate species status is considered questionable (Hansen *et al.* 2008). Although the Brown trout *Salmo trutta* is categorized as under "least concern" on the German red list of species this category does not reflect the conservation status of the anadromous migration type of *S. trutta* known as sea trout, which is subjected to marked declines in many populations within Germany (Kottelat & Freyhof 2007). Seven of the remaining eight anadromous fish species reach IUCN conservation criterion of near threatened or higher (Table I.1), while one species (*Alosa fallax*) has not been evaluated, due to its primary residence in marine waters (Freyhof 2009). Although none of the three catadromous species has been evaluated in the German red list of species (Freyhof 2009) (Table I.1), at least the European eel *Anguilla anguilla* can be considered of prime conservation concern, as it has suffered strong population declines throughout its entire distribution range since the 1980ies (Feunteun 2002) and is classified as critically endangered on a global basis (Table I.1). The situation in Germany is very characteristic for diadromous fish populations of both sides of the North Atlantic. Declines in North Atlantic diadromous species follow a common pattern (Limburg & Waldman 2009), characterized by a sharp population decline between the end of the 18th and the beginning of the 20th century (Figure I.1). This pattern was exemplified for several anadromous fish species of River Rhine, leading to the local extinction of the

European sturgeon (De Groot 2002), Atlantic salmon (Lelek & Köhler 1990), Allis shad (De Groot 1990) and also the North-Sea houting (Freyhof & Schoter 2005). Similar processes are found in Elbe River (Debus 1996; Monnerjahn 2011) and Oder River (Mamcarz 2000). In contrast to most anadromous species the catadromous European eel experienced the sharpest rate of decline throughout Europe since the 1980ies (Belpaire *et al.* 2009; Limburg & Waldman 2009).

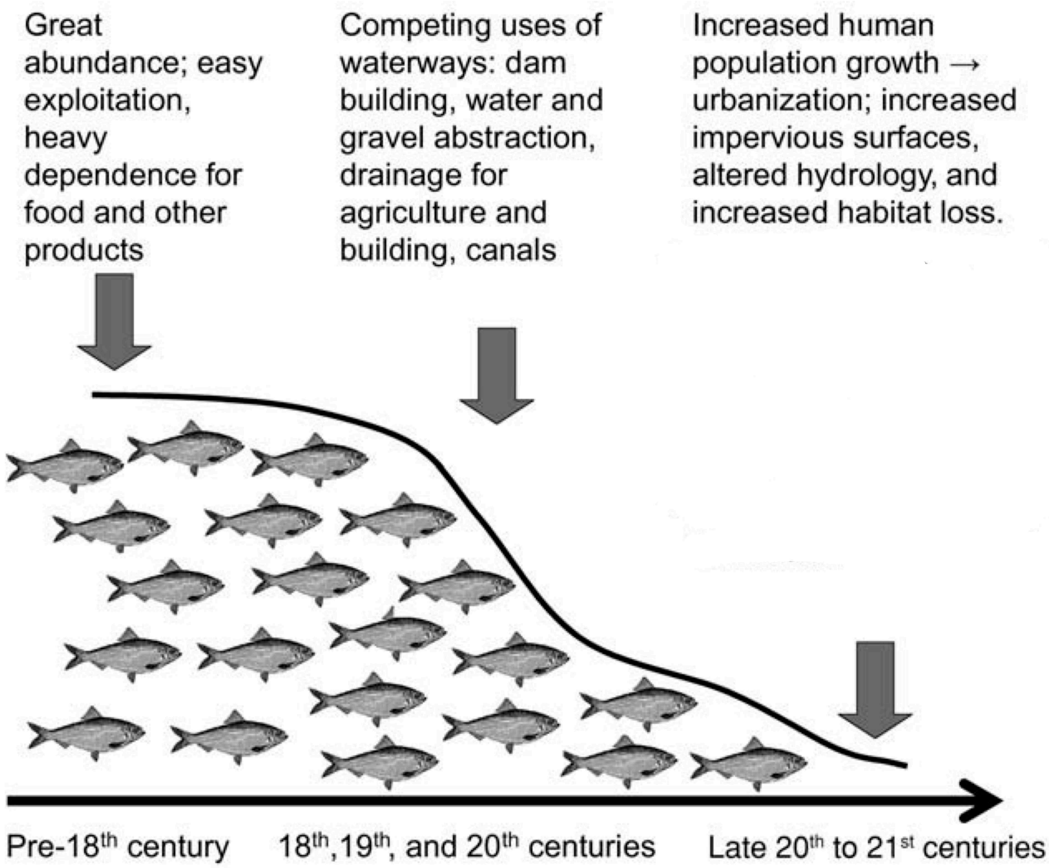

Figure I.1 Conceptual diagram of the general history and factors leading to declines in North Atlantic anadromous species. Most species were heavily exploited before industrialisation and physical alteration of waterways; further watershed alterations due to human population expansion and habitat loss (Modified from Limburg and Waldman 2009).

Although the management priorities for stocks of diadromous fish are to some extent species- or population-specific, most population declines can be attributed to the same causes, which have been summarized by Limburg & Waldman (2009). Limburg & Waldman (2009) specifically focused on conservation issues concerning diadromous fish species of the North Atlantic. Most of the stated reasons for population decline also apply to freshwater species in general, as summarized by Maitland (1995) and Helfman (2007). The three largest threats to diadromous species are considered to be the blocking and the destruction of spawning habitat, Overfishing and pollution and either one of those threats apply to most imperiled diadromous fish populations of the Northern Atlantic (Table I.2). Also other threats as genetic hybridisation with aquaculture escapees have been identified (Table I.2), causing strong declines of specific populations of diadromous fish (Limburg & Waldman 2009).

Despite the fact that the chemical water quality in Germany has substantially improved within the last decades overall ecological status of Germanys rivers can still be considered as mediocre or low compared to a natural state of river systems (BMU 2014). The impaired ecological condition is mainly connected to the high degree of river engineering of German river systems, as all major German rivers have been severely modified and over 90% of the federal waterways have “distinctively” or “completely” changed their structure from their natural river bed in order to improve their use as waterways for shipping traffic (BMU 2014). Due to various river modifications as channelization and the separation of vast parts of the rivers original floodplains by dykes, the structural heterogeneity and also the habitat suitability for many aquatic organisms has been severely diminished. Additional to the overall low degree of structural complexity, German river systems are interrupted every second kilometre on average by transverse technical structures of various types, influencing the biology and morphodynamic continuity of these systems and thereby affecting the behaviour and reproductive success of German freshwater and diadromous fish (BMU 2014).

Table I.2 Major threats for diadromous fish species.

Cause of decline	Explanation	Reference
Blocking and destruction of spawning and juvenile habitat	• Anadromous fish are hindered to reach their spawning grounds • Altered water discharge modifying behaviour • Decreased habitat availability due to dredging and channelization • Reduced availability of shallow water habitat reduces recruitment potential.	De Groot (2002) Helfman (2007) Limburg & Waldman (2009)
Overfishing	• Especially important in late maturing species, as sturgeons and species susceptible to fisheries at variable life-stages, as the European eel	De Groot (2002) Helfman (2007) Limburg & Waldman (2009)
Pollution	• Large amounts of raw or lightly treated human sewage and agricultural run-off lead to eutrophication and oxygen minimum zones blocking spawning migrations • Contaminants (e.g. PCBs, PAHs, heavy metals) induce sublethal effects. • Acidification from atmospheric deposition of contaminants Direct mortality due to large scale release of chemicals	De Groot (2002) Helfman (2007) Limburg & Waldman (2009) Cioc (2002)
Climate change	• Altering species distribution • Shifting life history patterns of anadromous fishes potentially disrupting established ecologic relationships • Decreased flows of spawning rivers in northern habitats	Helfman (2007) Lassalle *et al.* (2008) Limburg & Waldman (2009)
Presence of electric generating plants	• Direct cause of mortality during migration	Helfman (2007) Limburg & Waldman (2009)
Competition, disease transfer and genetic hybridisation with escapees from aquaculture	• Aquaculture in open containment facilities poses large problems particularly on salmon populations of Northern Europe, Canada and the United states	Naylor *et al.* (2005) Limburg & Waldman (2009) Helfman (2007)
Competition with non indigenous species	• The introduction of piscivorous species can change the predation regime of particular habitats • Competition for common resources	Helfman (2007) Limburg & Waldman (2009) Cucherousset & Olden (2011)

Degradation of River Rhine:

As the high degree of river engineering of River Rhine is unique among European rivers, River Rhine can be regarded as a perfect example for the degradation of a complex ecosystem to a streamlined shipping lane merely serving industrial purposes (Cioc 2002). Hydromorphological modification works started in 1817 with Gottfried Tulla's Rectification Project in the upper Rhine and was followed by several later river-engineering operations in all sections of the river. While Tulla's project focussed on flood control in the upper Rhine, most later engineers focused on the deepening of the river bed and the widening of the shipping lane and were more concerned with navigation than flood control (Cioc 2002). Due to insufficient international coordination, as well as the limited hydro-engineering expertise, many of the later projects were mere fixes of problems deriving from earlier river modifications to balance the often contradicting needs of navigation, flood-control, erosion-control, agriculture and hydroelectric power generation (Cioc 2002). All in all River Rhine lost approximately 90% of its floodplain during these successive engineering operations (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995), creating a system of reduced fish biodiversity and prone to severe flooding events (Cioc 2002; Brenner *et al.* 2004).

With regard to the conservation of diadromous fish the most devastating consequence of river modification can probably be deemed the creation of dams in River Rhine and its tributaries for navigational and hydroelectric power generation (Brenner *et al.* 2004). With a total number of 110 dams in 2010 the River Rhine system is still considered to be of highest priority among all German river systems with regard to re-establishing the linear passability for migratory fish (Scholten *et al.* 2010). Also fisheries played a detrimental role in the extinction or reduction of particular anadromous species in River Rhine, especially in the extinction of late maturing European sturgeon (Gessner 2000), but also in the decline of the Atlantic salmon (Monnerjahn 2011), Allis shad (De Groot 1990) and other anadromous species (De Groot 2002). However the second most important reason for the decline of diadromous fish species in River Rhine can be considered the widespread pollution of this system. Since the beginning of the 20th century the water quality of River Rhine was rapidly deteriorating caused by increasing discharges of industrial and agricultural production, as well as elevated discharges of domestic wastewater (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995; Cioc 2002). Water quality degraded to such an extent, that states bordering River Rhine were obligated to cooperate, resulting in the foundation of the International Commission for the Protection of the Rhine (ICPR) initiated by the Dutch in the 1950ies (Curlee 1999). Participants of this commission are France, Germany, Switzerland, Luxembourg and the Netherlands. Until the 1970ies chemical producers routinely disposed hazardous compounds directly into Rhine and its tributaries, as for example discovered after a massive fish kill near Bingen in 1969, which was caused by 40 to 50 Kg of Thiodan-related pollutants directly dumped into the River Main on a daily basis (Cioc 2002). Starting form the mid 1970ies agreements on emission reductions were successfully implemented and inorganic nutrients, organic compounds as well as heavy metal loads were effectively reduced, resulting in increased oxygen values as well as a measurable recovery of the biological community in the 1980ies (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995). However these improvements were followed by a giant setback, when in 1986 the Sandoz chemical spill directly killed several hundred tons of fish (in particular estimated 200 tons of eel) after the release of nearly 30 tons of agrochemicals, including insecticides, herbicides and fungicides (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995; Brenner *et al.* 2004).

Restoration of River Rhine:

The Sandoz spill unveiled the short-sighted disposal practices and insufficient safety measures practiced by the chemical industry for years (Cioc 2002) and stimulated a major ecological restoration project by the ICPR, the Rhine action programme (RAP) for ecological rehabilitation (Curlee 1999; Molls & Nemitz 2008). The main ecological goal of this project was the successful return of higher level migratory fish species and in particular the Atlantic salmon, but also focused on the general improvement of sediment and water quality of River Rhine and the associated North Sea. The reintroduction of the Atlantic salmon as envisioned in the Rhine Action programme required several measures before the return of this species could be considered a possibility.

The first step in order to allow the return of higher level migratory species was the improvement of water quality and all Rhine states agreed upon a pollution reduction of 50% until 1995, furthermore water quality targets were set for about 50 priority compounds based on values for drinking water supply (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995).

It is widely recognized that ecological rehabilitation of River Rhine can not be achieved by water quality improvements alone, but has to include a remediation of hydrological and structural alterations (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995). Therefore the Ecological Master Plan for River Rhine included the restoration of the mainstream of River Rhine and its main tributaries as habitats for migratory fish, as well as the protection, preservation and improvement of important reaches of River Rhine, in order to increase biodiversity of indigenous plants and animals (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995).

In the year 2000 a second legal instrument was put into place focusing on the ecological rehabilitation of European water bodies including river Rhine: the European water framework directive (WFD) of the European Union. The primary goal of the WFD is the achievement of a good ecological status of all European water bodies until 2015, applying an integrated approach for water management. A good ecological status is defined as only a slight deviation from natural conditions (Achleitner *et al.* 2005). With regard to the successful implementation of the WFD in Germany the restoration of hydromorphological damage, as well as the re-establishment of river continuity for fish and other organisms were identified as impairing the ecological status of German river systems. However also high nutrient input, leading to eutrophication, and specific contaminant inputs can be considered a problem in Germany (BMU 2013). Both of these legal frameworks the WFD and RAP paved the way for the return of the Atlantic salmon and other anadromous species.

Already in 1990 distinct improvements in water quality of River Rhine could be measured and two thirds of the target compounds reached concentrations below the set limits (Lelek & Köhler 1990). Although the limits for some heavy metals, chlorinated hydrocarbons and also inorganic fertilizers were not met, progress was clearly visible. Despite the acute consequences of the Sandoz accident, also the fish community showed a remarkable recovery, as 40 of the formerly 47 indigenous species of river Rhine were found in a survey in 1990 (Lelek & Köhler 1990). Between 1990 and 1995 a further reduction of priority substances between 50-70% percent was achieved and target values of the Rhine Action Programme were met (ICPR 2000).

Although further reductions in nutrient inputs could be achieved until the year 2012 within the focus of the WFD (BMU 2014), further efforts to improve the water quality of River Rhine are essential. Despite tremendous improvements in water quality River Rhine can still be regarded as one of the most polluted rivers in Europe (De Groot 2002) and particularly the re-mobilisation of PAHs, PCBs and heavy metals from contaminated sediment areas pose a large risk to the water quality of the main channel of River Rhine (ICPR 2014). Remobilisation of toxins from contaminated sediments has also also been shown to impose negative consequences for the fish community in connected oxbow lakes of River Rhine, which have to be considered important developmental and reproductive fish habitats (Heimann et al. 2011).

Measures to improve river continuity and hydromorphological condition of River Rhine started much later that the pollution abatement programme (van Dijk, Marteijn & Schulte-Wülwer-Leidig 1995). Coordinated by the ICPR several local projects in Rhine tributaries focused on the restoration of spawning grounds and feeding grounds for salmon and the improvement of general habitat quality, e.g. by reconnecting old river branches and flood plains to the main stream (Curlee 1999; Brenner *et al.* 2004). Probably the most straight forward approach of the Ecological Master Plan of River Rhine is the removal of obstacles for fish migration. Several projects were undertaken in this respect (Curlee 1999). Two of the most important of these projects can be considered the building of fish passes at the Rhine dams of Iffezheim and Gambsheim, which make it now possible for migratory fish species to reach Basel (Brenner *et al.* 2004). With regard to the reintroduction of salmon about 34 ha of suitable spawning grounds and approximately 323 ha of juvenile habitat have been identified within the project areas of the Rhine system (Molls & Nemitz 2008). Available habitat is only a minor fraction of the natural habitat space before the industrial development and much additional restorative action is necessary to provide conditions for the re-establishment of a self-sustaining population of salmon (Molls & Nemitz 2008).

Former Re-introduction projects of anadromous fish species in River Rhine:

The reintroduction of the Atlantic salmon was always one of the main priorities of the RAP and salmon was the first species to be reintroduced into this river system (Brenner *et al.* 2004; Monnerjahn 2011).

The reintroduction program had two goals, first of all to return this species to its natural spawning grounds and thereby supporting European salmon stocks, secondly as an indicator of the on-going river restoration efforts and to increase public acceptance of these measures (Molls & Nemitz 2008). Stocking operations of salmon started in 1988 in River Sieg. Between 1988 and 2003 about 19 million salmon were released throughout the River Rhine on German, French, Luxembourgian and Swiss territory. At the beginning of the programme mostly unfed fry were being stocked while later to some extend also older parr and smolt were stocked in a multiple life stage release strategy (Molls & Nemitz 2008). Already in 1990 two years after the initial fry release the first adult salmon-returners were detected in River Sieg and in 1994 the first natural reproduction of salmon was observed in the same river (Molls & Nemitz 2008). Although from 1990 to 2003 2,436 salmon were detected in the whole Rhine catchment, the successful re-establishment of a self-sustaining population has to be considered far from being complete, as the return rates of salmon are extremely low compared to those of natural populations (Molls & Nemitz 2008). The future re-establishment of this species relies on additional restoration actions, as the further improvement of spawning areas, the opening of upstream and downstream migration routes and the control of agricultural run-off (Molls & Nemitz 2008). Due to the sustained dependence on stocking operations the ICPR prolonged the period for achieving the goal of self-sustaining salmon populations until the year 2020 within the project "Rhine 2020" as promoted in 2001 (ICPR 2007).

Despite not achieving the primary goal of creating self-establishing population of salmon in river Rhine, the RAP paved the way for other anadromous species.

Concerning seatrout, the anadromous form of *S. trutta,* stocking operations have to be clearly separated from those of the Atlantic salmon *S. salar*. Although seatrout also suffered from the impacts of river engineering and pollution, this species has never been extinct in River Rhine (Bij de Vaate *et al.* 2003). Therefore the stocking operations performed particularly in the Mittelrhein (e.g. Sieg, Wupper, Ahr, Saynbach and Lahn) have to be considered supportive stocking measures for an original Rhine population of seatrout or for a population of naturally recolonizing specimens from adjacent river systems and are to my knowledge exclusively based on naturally returning adults (Schreiber & Diefenbach 2005).

The second species to be re-introduced in River Rhine after it became extinct within this system in the 20th century (Kranenbarg, Winter & Backx 2002) is the North Sea houting *C. oxyrinchus* (Borcherding *et al.* 2010). Although wide disagreement with regard to the exact identity of the species exists (Freyhof & Schoter 2005; Dierking *et al.* 2014) I will continue to use the name North Sea houting for the anadromous coregonid species occurring in river Rhine that according to literature is either to be termed *C. maraena* or *C. oxyrinchus* (Hansen *et al.* 2008). The situation is further complicated by the occurrence of at least a second population of (non-migratory) coregonids (De Groot 2002), which have been shown to most likely origin from Lake Constance (Freyhof, Pohlmann & Weibel 2012). Between 1996 and 2005 approximately 1.9 million North Sea houting originating from the Danish river Vida were stocked in two locations of the lower Rhine (Borcherding *et al.* 2010). Already in 2006 only a minor proportion of these Nort Sea houting was assessed to derive from stocking operations and therefore must origin from natural reproduction (Borcherding *et al.* 2010). The re-introduction of North Sea houting can be considered a huge success and stocking operations only lasted 9 years and were ceased due to the successful establishment of a self sustaining population (Borcherding *et al.* 2010; Borcherding 2011).

Also the re-introduction of the European Sturgeon *A. sturio* has been considered in River Rhine, which used to be the second most important river system for this species in Germany after the Elbe river (Nemitz 2010). Despite limiting factors as intense navigation, residual pollution levels and fisheries, River Rhine has been evaluated to be principally suitable for the re-introduction of this species. Initially the re-introduction should focus on stretches of the lower Rhine and the Rhine-delta. However due to the fact that the population is limited to only few specimens and the long maturation time of this species, stocking material is not available for the next years. The restoration of *A. sturio* in river Gironde and the re-introduction in river Elbe have to be considered of higher priority and are essential for the survival of this species (Nemitz 2010).

The Allis shad: General biology and re-introduction efforts at River Rhine:

The most recent attempt to re-introduce an anadromous fish species in River Rhine has been performed with the Allis shad *A. alosa.*

As already mentioned *A. alosa* can be considered as one of the most important fisheries resources of River Rhine in the 19th century, with more than 250,000 individuals being caught annually only in the Netherlands (De Groot 1990). The original distribution range of this species stretched from the southern part of Norway to Morocco (Figure I.2) and is now limited to few river systems in France and Portugal (Figure I.2, Bagliniere *et al.* 2003). Only five rivers in Europe are currently supporting functional nonresidual stocks (France: Charente, Dordogne, Garonne, Adour, Portugal: Mondego).

A. alosa spawns in the middle to upper reaches of European river systems. Juveniles migrate to sea within their first year and adult Allis shad return 3 – 8 years later, the majority of females matures at ages between 5 and 6 years, while males typically mature between 4 and 5 years. (Aprahamian *et al.* 2003). The timing of spawning migration of this species is dependent on latitude and occurs between December to August, population of the south migrating earlier in the year than populations in the northern distribution range (Aprahamian *et al.* 2003, Bagliniere *et al.* 2003). Water temperature has a strong influence on spawning behaviour and migrational activity only takes place at temperatures ranging from 12-20 °C (Bagliniere *et al.* 2003). Migrational behaviour is also influenced by other factors such as sex, flow rate and tidal state (Aprahamian *et al.* 2003, Bagliniere *et al.* 2003). Spawning of *A. alosa* generally occurs between 16 to 18 °C at night and is characterized by specific behavioural sequences, such as rapid circular swimming at the water surface, thereby emitting splashing sounds known as the "bull phenomenon" (Bagliniere *et al.* 2003). Specific fecundity of female Allis shad is high, ranging between 59,500 eggs towards the southern distribution range and 236,300 eggs in the Gironde-Dordogne-Garonne-basin (Aprahamian *et al.* 2003); therefore single female specimens can produce well over 400,000 eggs. After spawning eggs (1-2 mm diameter) drift in the current before becoming embedded in the substrate, where larvae hatch 4-8 days later (Bagliniere *et al.* 2003).

Apart from the observation that larvae of *A. alosa* exhibit a nektonic behaviour until 36 dph (Veron, Jatteau & Bardonnet 2003) few details about the larval and earliest juvenile phase prior to their downstream migration can be obtained from scientific literature.

Seaward emigration begins within the first year of life and takes place in schools for a duration of 3-6 months (Bagliniere *et al.* 2003). Factors influencing downstream migration are temperature, river discharge and also biological factors, such as size and salinity tolerance (Bagliniere *et al.* 2003). In the Gironde estuary Allis shad arrive at 88 dph on average and stay for approximately 11 days before entering the Atlantic ocean (Lochet, Boutry & Rochard 2009). In the estuary juvenile Allis shad are able to utilize a variety of food sources including aquatic insect larvae, molluscs, zooplankton and mysidae (Bagliniere *et al.* 2003). Few data are available about the marine phase of Allis shad, however most observations reveal that they remain in schools on the continental shelf in depths of 70 - 300 m and predominantly feed on zooplankton (Bagliniere *et al.* 2003).

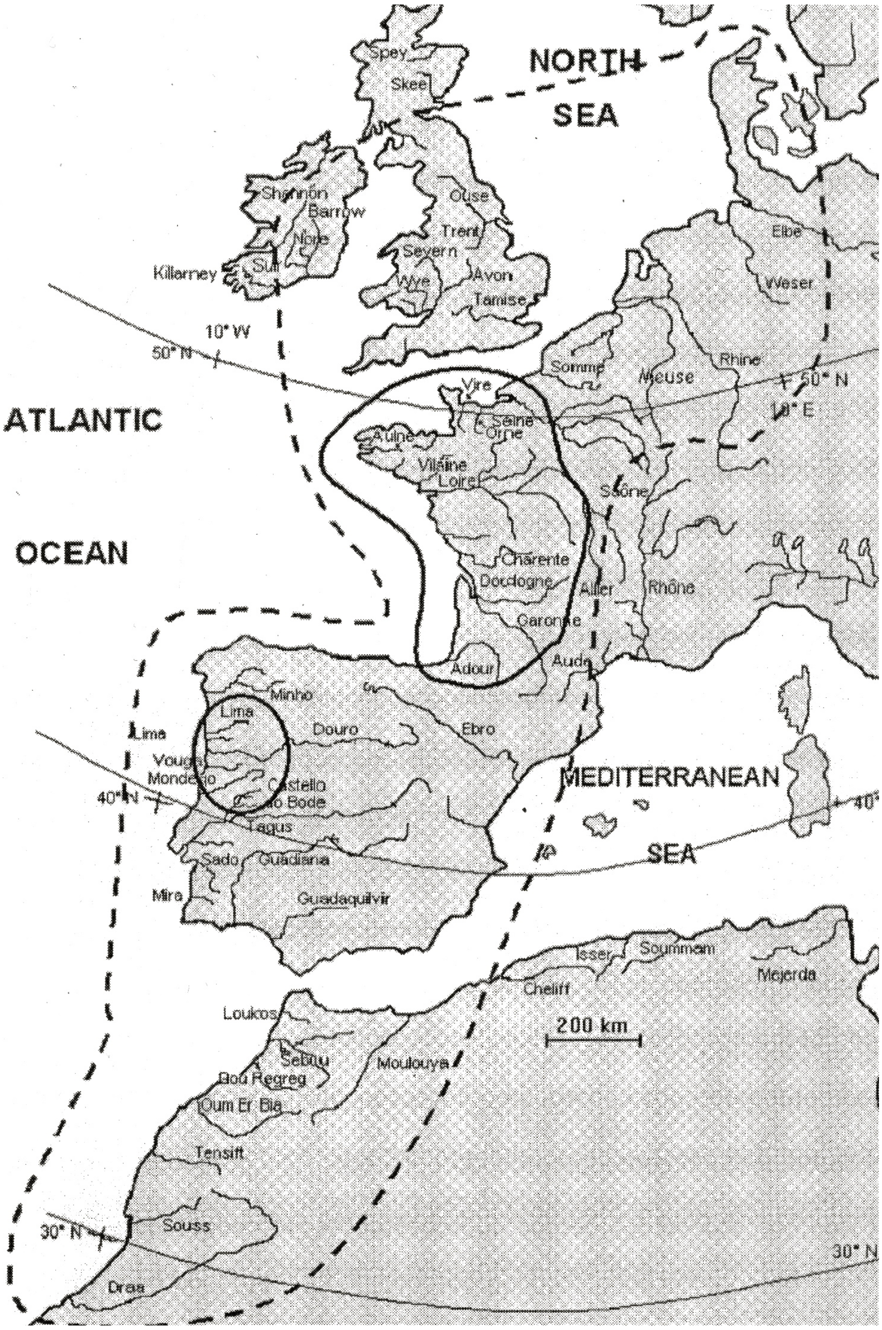

Figure I.2: Historic (dashed line) and current (solid line) distribution of *A. alosa.* Main Rivers colonized at the end of 19th, beginning of 20th and beginning of the 21st century are shown. From Bagliniere et al. (2003).

Starting in 2003 the general feasibility of a re-introduction project of Allis shad in River Rhine was assessed with regard to biological, as well as socio-economic aspects (Beeck 2004). Based on the IUCN guidelines on the re-introduction of species (IUCN 1998) several criteria are essential for the successful re-introduction of a species. Those criteria were assessed for the Allis shad in a feasibility study (Appendix A.1) and initial investigations about the aquaculture were started.

Marking procedures for this species were developed by German and French institutes, in order to design a specific management concept (Beeck 2004).

After the positive evaluation of the feasibility study and promising experimental results regarding the propagation and chemical marking of this species an EU-funded LIFE-project "The re-introduction of Allis shad (*A. alosa*) in the Rhine System" was started in 2007. During the project duration of three years the knowledge gained on shad rearing during feasibility studies of the French institute CEMAGREF was implemented by the project partner MIGADO in the first European fish farm for Allis shad in Bruch (France) (Klinger 2011). The full production cycle from catching upstream migrating shad in rivers Garonne and Dordogne until stocking of Allis shad larvae in River Rhine can be separated into the following 4 steps (Table I.3, Figure I.3):

Table I.3 The production cycle of *A. alosa* from the catch of mature individuals until stocking in River Rhine.

1. Monitoring & Catch	Number of upstream migrating mature individuals is assessed via the monitoring station of specialized fish elevators located at the Golfech dam at River Garonne and the Tulieres dam at the River Dordogne. Identified Allis shad individuals are separated from the outlet of the monitoring station and then transported alive in a special tank to the LIFE Allis shad hatchery in Bruch (Clave 2011).
2. Aquaculture	*a) Induced spawning:* At the hatchery mature individuals are transferred to 10000 l spawning tanks at a sex ratio of 2:1 or 3:2 (male:female), where they are being injected 100µg/kg synthetic luteinizing hormone-releasing hormone (LHRAa), to stimulate spawning. As females and males are kept in one tank eggs are fertilized directly in the water column of the spawning tank. Spawning of eggs is performed in several portions and eggs are automatically collected in an egg collector system connected to the drainage of the tank (Clave 2011).
	b) Egg incubation: Eggs are transferred into custom built upwelling incubators and incubated at approximately 18 °C (Clave 2011). Upon reaching the eyed egg stage they have to be transferred to special hatching jars, from where larvae can directly attain the rearing tank subsequently to hatching.
	c) Larval rearing: After approximately 4 days at 18°C yolk sac larvae of Allis shad hatch from the eggs and swim to the outlet of the hatching jar and reach the larval rearing tank. From 1 dph Allis shad larvae are fed a combination of *Artemia* nauplii and microparticulate diet in a co-feeding regime (Clave 2011).
3. Marking	Prior to the transport to Germany for stocking Allis shad larvae are immersed in a solution of 300 ppm Tetracycline hydrochloride for 4 hours in order to chemically mark the otoliths of these fish, for later identification of returning stocked adults (Jatteau 2010).
4. Stocking	Larvae are transported in water filled oxygenated plastic bags to the stocking site at River Rhine and transferred into flow through tanks, to allow adaptation to ambient conditions. To ensure good nutritional status prior to stocking they are fed with *Artemia* nauplii.

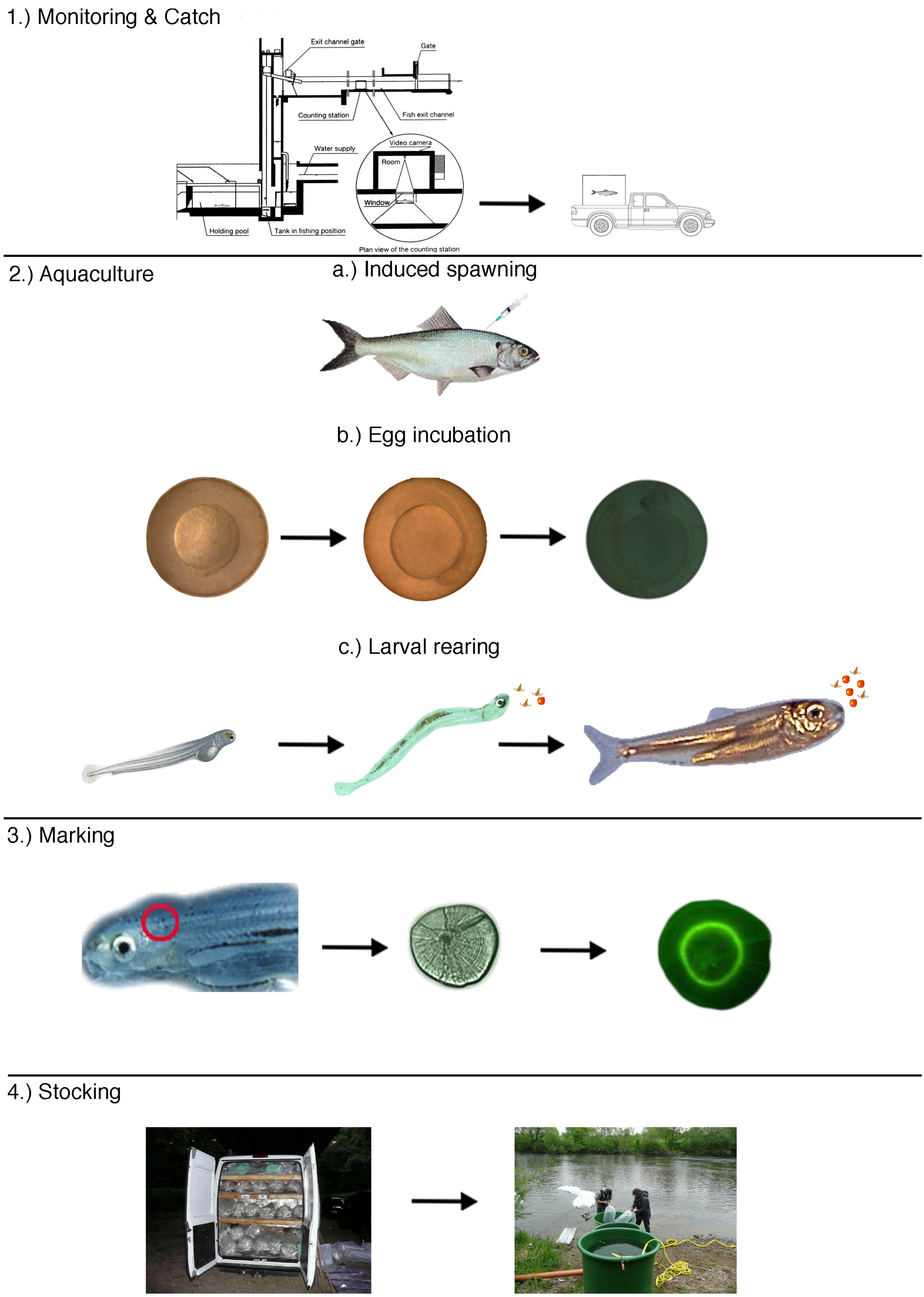

Figure I.3 The production cycle of *A. alosa* from the catch of mature individuals until stocking in River Rhine. Modified from Clave (2011) and Jatteau (2010).

During the project duration of 3 years a total of 4.8 million Allis shad larvae were reared according to the stated methods and stocked in river Rhine in years 2007 to 2010. To resume the stocking measures and monitoring operations in River Rhine a second LIFE project (LIFE+) was initiated in 2011. Additionally to the continuation and improvement of previous LIFE measures, the LIFE+ project “Conservation and restoration of the Allis shad in the Gironde and Rhine watersheds” focuses on the establishment of an EX-situ stock for Allis shad, the understanding of potential bottlenecks in recruitment in the Gironde watershed and the optimization of fish pass design for upstream migrating shad in River Rhine (Scharbert 2011a).

Objectives:

The overall goal of this thesis is to demonstrate that extinct local populations of Allis shad can be restored in river systems throughout this species historical distribution range by artificial propagation and to determine which factors can be considered critical in that respect.

The first part of this thesis deals with the question, how *aquaculture and stocking* practices for Allis shad and other endangered anadromous fish species can be optimized, while the second part focuses on the topics of *conservation* and *restoration* of Allis shad in River Rhine and other European rivers. Regarding the improvement of *aquaculture and stocking* practices of *A. alosa* the larval rearing period and the establishment of an Ex-situ stock lay within the scope of this thesis. Chapter II and chapter III aim at the development of a cost effective, easy to use source of life food for fish larvae. As mass marking practices are an integral part of stocking measures of Allis shad in River Rhine, I investigated water-hardness related mortality during emersion marking of the model-species zebrafish (*Danio rerio*) with Oxytetracycline-hydrochloride (Chapter IV). Chapter V focuses on the aetiology of deformations in Allis shad under captive rearing conditions and points to strategies preventing the development of these deformations in the future. With regard to the *conservation and restoration* of Allis shad the physiological temperature sensitivity of this species was evaluated and discussed in relation to *A. alosas* widespread disappearance throughout Europe, which has also been attributed to climate change (Chapter VI). Chapter VII focuses on the *restoration* of the most important historical population of this species in River Rhine and gives an overview about the success of stocking operations and advancement of monitoring measures within the framework of the LIFE-project (Figure I.4).

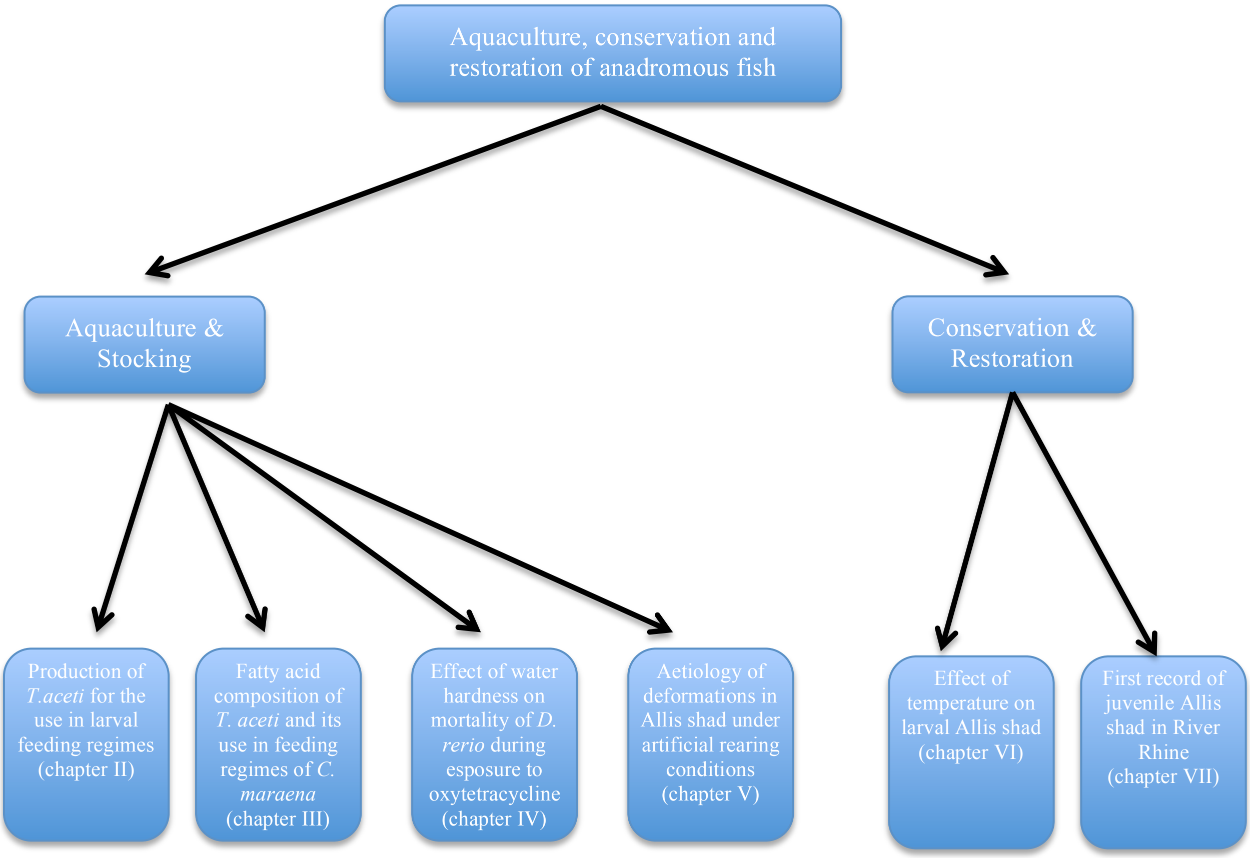

Figure I.4 Overview of the different chapters assigned to the two topics of my thesis.

Chapter II: Improving nematode culture techniques and their effects on amino acid profile with considerations on production costs

Bela H. Buck, Jens Brüggemann, Matthias Hundt, Adrian A. Bischoff, Wilhelm Hagen

(***Journal of Applied Ichthyology* (2015)**; volume 31, issue 1, pages 1-9.)

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http://onlinelibrary.wiley.com/doi/10.1111/jai.12645/abstract

Chapter III: Fatty acid composition of *Turbatrix aceti* and its use in feeding regimes of *Coregonus maraena* (Bloch, 1779): is it really a suitable alternative to *Artemia* nauplii?

Matthias Hundt, Jens Brüggemann, Adrian Alfred Bischoff, Dominik Martin-Creuzburg, Rene Gergs, Bela H. Buck

(***Journal of Applied Ichthyology* (2015)**; volume 31, issue 2, pages 343-348.)

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http://onlinelibrary.wiley.com/doi/10.1111/jai.12668/abstract

Chapter IV: Effect of water hardness on the mortality of zebrafish (*Danio rerio)* during exposure to Oxytetracycline

Matthias Hundt, Benjamin Schreiber, Rainer Eckmann, Bjørn Tore Lunestad, Hannah Wünneman, Ralf Schulz

(**Bulletin of Environmental Contamination and Toxicology (2015)**; online only publication)

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http://link.springer.com/article/10.1007%2Fs00128-015-1699-x

Chapter V: First Report on cranial deformations of Allis shad (*Alosa alosa*) reared in captivity.

H. Wünnemann, S. M. Bergmann, U. Eskens, A. Scharbert, M. Hundt, M. Lierz

(Brief communication, In preparation)

Abstract

The Allis shad, *A. alosa*, an anadromous fish of the family *Clupeidae* is an endangered European species. In order to re-establish Allis shads in the river Rhine, which formerly housed one of the greatest and most important populations of *A. alosa* in its native range the EU-LIFE-Project „The re-introduction of Allis shad *(A. Alosa)* in the Rhine system“ was started in 2007 by releasing shad larvae bred from genitor fish of the Gironde-Garonne-Dordogne-population, the biggest remaining population of the Allis shad. In order to get independent from the wild stocks for breeding in the future, a pilot facility for an ex situ population stocked with juvenile Allis shads from France was established in 2011. At an age of 1 month up to 100% of these fish showed approximately 0.5-0.8 cm large, fluid filled, transparent cysts on their heads in conjunction with the lower jaw. These deformities were so far not described in the literature. The performed bacteriological, virological, parasitological and histological examinations did not detect possible infectious agents. In conclusion environmental or nutritional factors are considered causal for the development for these deformations, and have to be regarded a risk for the establishment of an ex-situ stock of Allis shad.

Brief communication

The Allis shad (*A. alosa*, Linné 1758) is an anadromous fish of the family *Clupeidae* that originally occurred along the eastern Atlantic coasts of Europe and northwest Africa. The populations of Allis shads decreased severely by the middle of the 20th century. A combination of anthropogenic factors, such as over-fishing, increasing pollution, the construction of obstacles to migration and the destruction of spawning grounds were considered to be causal (Bagliniere *et al.* 2003). Over the past decades the continuous improvement of the environmental conditions within the Rhine ecosystem was a prerequisite for the reintroduction of the Allis shad. To prevent the progressive decline of the residual populations in the original native range and in order to reintroduce the species in one of its formerly most important river systems the EU-LIFE-Project „The re-introduction of Allis shad *(A. Alosa)* in the Rhine system" was started in 2007. Within the course of this project 10.66 million Tetracycline marked fry originating from adult Allis shads of the French donor population were released into the Rhine system in yearly intervals. In context of the follow up EU-LIFE+-Project "Conservation and restoration of the Allis shad in the Gironde and Rhine watersheds" a pilot *ex situ* stock plant in Aßlar, Germany in order to establish of a Broodstock was started in order to get independent from the wild populations for stocking purposes in the future (Scharbert et al. 2011b). Although breeding techniques for other migratory fish species, e.g. several salmon and sturgeon species have been developed and are already well established due to the commercial value of these species (Bjørndal 1990; Bronzi, Rosenthal & Gessner 2011), the experience in the aquaculture of the Allis shad is rather limited. The Aquarium of La Rochelle (France) started breeding trials with this species (Jatteau *et al.* 2013). A large fraction of juveniles of each generation displayed distinct cysts in the cranial region. The reasons for the development of those cysts remain unclear, whereby the diet was assumed possibly causative but was not further investigated (Jatteau *et al.* 2013). In Aßlar, Germany, nearly all Allis shad displayed large cysts in the head region at an age of approximately 1 month. Additionally other severe deformations of the shape of the head, the jaw, the opercula and the fins were observed. To date the cysts are a common problem in bred Allis shads and are likely increasing larval mortality (Jatteau *et al.* 2013), as well as affecting the visual appearance of juvenile and adult fish. Therefore they are considered to be an important risk for the success of the reintroduction program. Although a variety of deformations of wild and farmed fish have been observed and attributed to a number of factors (Barahona-Fernandes 1982; Madsen & Dalsgaard 1999; Cobcroft *et al.* 2001; Sandland & Goater 2001; Lall & Lewis-McCrea 2007; Osman *et al.* 2007; Jezierska, Ługowska & Witeska 2008; Boglione *et al.* 2013) this is the first report of such type of cysts in fishes including an analysis of possible causative agents.

In Bruch, France, spawned eggs from the donor population were disinfected with Hydrogen peroxide 250 ppm for 10 minutes, transferred to incubators and repeatedly treated with Hydrogen peroxide 100 ppm for 90 minutes twice a day. After hatching the fry were kept in 600l tanks supplied with water from the "Canal du Midi" which was treated with mechanic filters and disinfected with UV. The fish were fed with decapsulated *Artemia nauplii* (Lavens & Sorgeloos 1996) and commercial fry feed (Caviar 100-200 μm and 200-300μm, Bernaqua, Belgium) during the light period. Temperature and oxygen (PCE- PHD-1, PCEinstruments, Meschede, Germany) were controlled daily (O_2: 6.5-8.5/l, Temperature: 19.5 to 25.3 °C). At the age of 5 days larvae were immersion-marked in Oxytetracycline hydrochloride (300ppm, exposition time 4 hours) (Jatteau 2010) before transporting them to Aßlar, (Germany) between 5-21 dph (Clave 2011). The breeding facility in Aßlar is equipped with a recirculating Aquaculture system (total volume 6m^3) consisting of 4 tanks (each 0.75m^3) and a filter system consisting of a swirl separator for particle filtration, a moving bed biofilter and UV-lamps for disinfection. They were fed with decapsulated *Artemia nauplii* (Micro Artemia cysts, Ocean Nutrition Europe, Essen, Belgian) (Clave 2011) and commercial fry feed (Perla Larva Proactive 6.0, Skretting, Stavanger, Norway) once an hour during the light period.

At an age of 1 to 2 month the affected batches showed approximately 0.5-0.8 cm large cysts on the head with prevalences up to 100%. They were in most cases located on the right side next to the nostrils, smooth, transparent and filled with a clear liquid (*Figure V.1*).

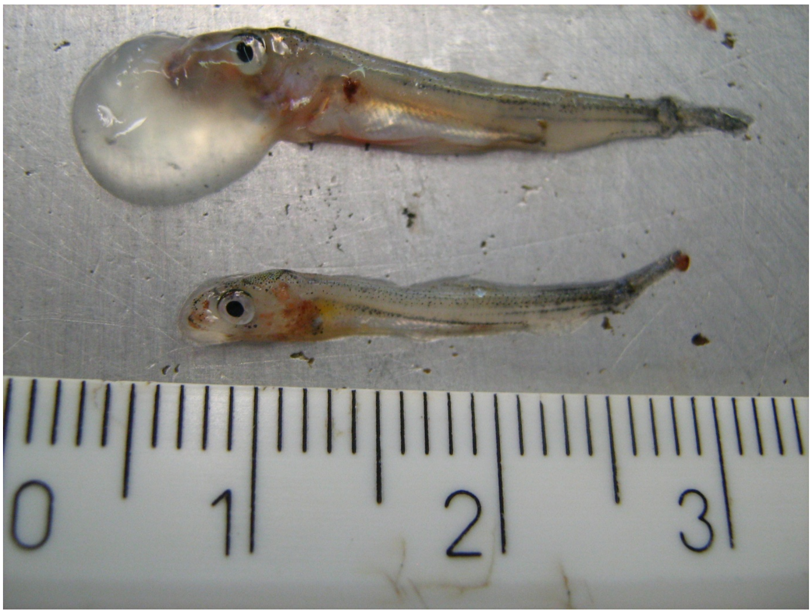

Figure V.1 2 month old Allis shads showing liquid filled, transparent cysts in the head region.

Some of the affected juveniles were euthanized with MS 222 (3-Aminobenzoic acid ethyl ester-methanesulfonate, SERVA Electrophoresis GmbH, Heidelberg, Germany) and necropsied using a binocular (KL 1500 electronic, Leica, Wetzlar, Germany). A bacterial examination was conducted on 5 Allis shads by drawing the fluid out after disinfecting the skin with ethyl alcohol with a sterile 13 gauge needle and 1ml syringe. The fluid was spread on Columbia agar with 5% sheep blood, Gassner-Agar and modified Shieh-Agar and incubated aerobically at room temperature for 5 days. Squash preparations of the cysts were checked under light microscopy (Zeiss, Axiostar plus, Oberkochen, Germany) for parasites. Furthermore 5 Allis shads were used for virological examination and therefore were pooled, homogenized using mortar, pestle and sterile sand, diluted in 10ml of minimum essential medium (MEM No. 5) with 0,01ml 10% Enrofloxacin and incubated at 4 °C overnight. Thereafter the sample was centrifuged for 30 min at 5000xg at 4 °C and 1ml supernatant was inoculated into 24 h old EPC, FHM, CCB and RTG-2 cell cultures in 25ml cell culture flask as duplicate. Inoculated cells were incubated for 24h at 26 °C, afterwards at 20 °C and examined for cytopathic effect (CPE) or possible toxicity in an inverted microscope on 3, 5 and 7 days post infection (dpi). On 7th dpi, the first passage (1p) cultures were subjected to 1 freeze/thaw cycle. The cell culture lysates from the samples in duplicate were pooled and 1ml supernatant was inoculated into fresh cells. Samples which caused no cytopathic effect after the third passage were considered negative. DNA-extraction was conducted from the remaining pellet after homogenization and centrifugation by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A semi-nested PCR (Bergmann et al. 2010), a PAN-Alloherpesvirus-PCR (Engelsma et al. 2013), combined with a nested PCR as well as a Iridovirus-PCR (Ohlemeyer et al. 2011) were performed. Another 5 juveniles were in whole fixed in 4% phosphate-buffered formaldehyde, dehydrated in an alcoholxylene series and embedded in paraffin. The fish were sectioned (4μm), stained with haematoxylin and eosin (H&E) and analysed using a Leitz microscope (Diaplan, Leitz, Wetzlar, Germany).

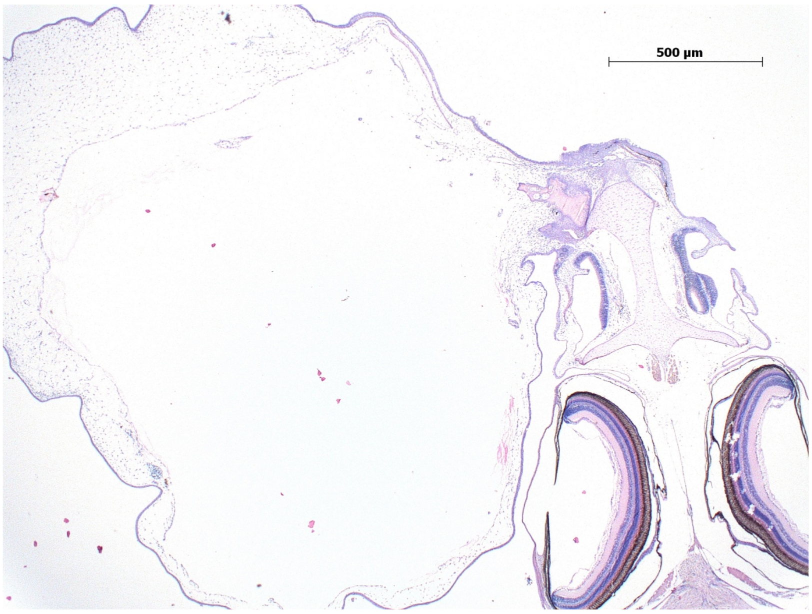

Figure V.2 Transversal cut through the head of a juvenile Allis shad. The cysts consisted of a cavity lined with a single layer epithelium, which was enclosed by loose connective tissue and an outer cover with epidermal epithelium. At the base of the cyst an irregular contoured piece of bone could be seen (Hematoxylin-eosin-stain, low magnification).

Figure V.3 At an age of approximately 8 month Allis shad display multiple severe deformations on the head, the lower jar and the operculum.

In the histological examination Allis shad showed thin walled cysts developing from the tissue of the upper jaw (Figure V.2). The cysts consisted of a cavity lined by a single layer epithelium, which was enclosed by loose connective tissue and an outer cover with epidermal epithelium. At the base of the cyst an irregular contoured piece of bone could be seen. No further pathological changes were detected in all other locations of the fishes. The parasitological and the bacteriological examination were negative. All performed PCRs were negative and the cell cultures showed no cytopathic effect (CPE) after three passages. Altogether the mortality was with 85% extremely high during the first. In the following month the cysts turned white, smaller and got a firm consistence and after 5 month just 5% of the fished survived. About half a year later there were just 0.2 cm large, white, solid extension remaining. At this time the Allis shads showed additionally multiple severe deformations of the upper and lower jaw, the head bones, the opercula and the fins (Figure *V.3*). Inspections of Allis shad carcasses which were available due to a short circuit in the recirculation system indicated the proportions of skeletal deformations. Within the 1+ cohort (n = 376) 95.5% and 79.2 % of the 2+ cohort (n = 48) of specimen exhibited these skeletal malformations.

Considering the various causes of deformations dealt with in the literature (Barahona-Fernandes 1982; Madsen & Dalsgaard 1999; Cobcroft *et al.* 2001; Sandland & Goater

2001; Yokohama *et al.* 2004; Lall & Lewis-McCrea 2007; Osman *et al.* 2007; Jezierska, Ługowska & Witeska 2008; Boglione *et al.* 2013) it is difficult to identify single causative factors for the cysts development. Infectious diseases as being causal for the development of the cysts have been excluded by use of bacteriological, parasitological, virological and histological methods. The pathogenesis of the cysts remains unclear but as the cysts caused the deformation of the head bones of the upper jaw at its base this skeletal deformation may be the origin for the development of the outer cyst. As causative factors for various forms of skeletal deformations in fishes chemical substances, genetic and environmental factors or an inappropriate nutrition are described (Boglione *et al.* 2013). Various substances, including heavy metals are known to cause deformation in fish (Shaklee *et al.* 1977; van den Brandhof & Montforts). As the water supply of the Aßlar breeding facility is directly derived from the wellspring, any kind of contamination seems unlikely. Furthermore the same effects have been observed in France in water originating from a separate source. Therefore contamination of the water source with chemical substances seems improbable. As larvae of each stocking event are originating from wild-caught mature individuals, genetic factors can likewise be considered unlikely reasons for the development of skeletal deformations, as these are mainly found in intensively produced fish with high inbreeding levels (Afonso et al. 2009). In 2012 approx. 2000 larvae at an age of 28 dph were transferred to a 200 m^3 cement fishpond at the University of Koblenz-Landau (Germany). The fish were kept only with natural feed from June 2012 until October 2012 After harvesting 34 fishes were morphologically analysed and none showed any deformation (unpublished data, Matthias Hundt). These fish were derived from the same batch of eggs, as fish being stocked at the Aßlar breeding station. The most likely factors responsible for the development of the stated malformations can probably be considered the nutrition of juveniles, as well as environmental factors regarding the rearing habitat, such as stocking density, hydrodynamics/water turbulence/water supply rate, rearing methodologies, light regimes, mechanical factors, levels of O_2/CO_2, pH, physical trauma/mechanical stress, salinity variation, tank characteristics and variation of temperature (Boglione et al. 2013). A possible influence of those environmental factors on the deformations remains to be further investigated. Likewise an unbalanced food in matters of different contents of various kinds of proteins, amino acids, lipids, vitamins, minerals and trace elements is described to cause multiple deformations in fish (Lall & Lewis-McCrea 2007; Boglione *et al.* 2013). The use of *Artemia nauplii* and microparticulate dry food is common practice in the production marine fish fry (Lavens & Sorgeloos 2000; Cahu & Zambonino Infante 2001). Many feeding protocols for other *Alosa spp.* (Leach & Houde 1999; Navarro, Carrapato & Ribeiro 2014) use Artemia nauplii enriched with a solution of polyunsaturated fatty acids, vitamins, minerals and free amino-acids, instead of decapsulated freshly hatched nauplii in combination with microparticulate feeds. In conclusion the observed deformations can be considered a severe sign of impaired health and welfare of Allis shad reared in captivity. Therefore potential causative factors need to be addressed urgently in order to ensure the successful establishment of an ex-situ broodstock which is considered to be detrimental for the long-term survival of this severely endangered fish species (Scharbert et al. 2011a).

Chapter VI: Effect of temperature on growth, survival and respiratory rate of larval Allis shad *Alosa alosa*

Matthias Hundt, Melanie Schiffer, Benjamin Schreiber, Monika Weiss, Cornelia M. Kreiss, Ralf Schulz, Rene Gergs

(***Knowledge and management of aquatic ecosystems* (2015)**; volume 416, issue 27, pages 27 p1 -27 p14**)**

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Chapter VII: First evidence of natural reproduction of the Allis shad *Alosa alosa* (L. 1758) in River Rhine following reintroduction measures

Matthias Hundt, Andreas Scharbert, Uwe Weibel, Götz Kuhn, Kathrin Metzner, Philippe Jatteau, Alisa Pies, Ralf Schulz, René Gergs

(***Journal of Fish Biology* (2015);** Volume 87, Issue 2, pages 487–493)

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Chapter VIII: General Discussion

Implications for the *aquaculture and stocking* of *A. alosa*:

So far it is not clear whether the reason for the observed deformations in Allis shad occurring at 1 month post hatch (Chapter V) can be found in the applied feeding regime. It has been demonstrated that nutritional inadequacies as for example the suboptimal supply with essential fatty acids at the onset of exogenous feeding and in the larval rearing period can be the reason for various malformations in the development of several fish species (Cahu, Zambonio-Infante & Takeuchi 2003, Lall & Lewis-McCrea 2007, Holt 2011), in particular the development of opercular deformities, as they have been observed in the majority of Allis shad (Chapter V) has been connected to a lack of DHA (Gapasin *et al.* 1998). The currently applied co-feeding regime for Allis shad by Clave (2011) consists of non enriched *Artemia* nauplii in combination with a formulated microdiet for marine fish larvae from day one post hatch. However it has been shown that the closely related American shad (*A. sapidissima*) is not capable of digesting formulated diets prior to 23 dph and it is not considered advisable to perform feeding of formulated feeds prior to 19 dph for this species (Hong *et al.* 2013). Therefore the application of an *Artemia*/microdiet co-feeding regime as early as one dph for *A. alosa* (Clave 2011) does not seem feasible. The uptake of essential nutrients via the microdiet might be restricted due to insufficient digestive capabilities at this early developmental stage which could potentially lead to the observed malformation in later life stages (Chapter V). Considering the fact that Allis shad larvae are usually released into River Rhine prior to 21 dph and sometimes already at 5 dph (Chapter VII), it should be evaluated whether *A. alosa* can effectively assimilate the fed microdiet prior to release. Furthermore it has to be considered if the feeding mode exhibited in captivity could potentially impair effective feeding behaviour in the wild, as it has been described for several salmonid species reared on formulated diets and later exposed to wild prey (Huntingford 2004). While feeding protocols developed for the introduction of larvae into an ex-situ Broodstock should be designed to facilitate weaning of larvae to formulated feeds, protocols for Allis shad larvae aimed for stocking should not only optimize nutrient provision, but also prevent the development of behavioural domestication effects. One way of mitigating behavioural constraints of captive breeding is the use of a larger variety of life prey items (Jackson & Rakocinski 2014). Jackson & Rakocinski (2014) demonstrated that the introduction of novel life prey items into the feeding regime of spotted seatrout could facilitate feeding success upon release. Integrating *T. aceti* as a vector of n-3 PUFA in an *Artemia* feeding regime as demonstrated for *C. maraena* (Chapter III) may therefore not only enhance growth and the provision with essential fatty acids, but also simultaneously increase behavioural flexibility of shad larvae in the wild. As the supplementation of *Artemia* nauplii with *T. aceti* has been shown to be beneficial only within the earliest rearing period of larval *C. maraena* (Chapter III), also the use of n-3 PUFA *Artemia*-enrichment techniques should be evaluated in detail for *A. alosa*. It has been demonstrated for several fish species, that enrichment of *Artemia* nauplii with n-3 PUFA in combination with different micronutrients can not only decrease the incidence of various malformations (Cahu, Zambonio-Infante & Takeuchi 2003), but also improves resistance against environmental stress factors (Kolkovski *et al.* 2000; Noori *et al.* 2011a), as commonly experienced during transportation and stocking. Therefore feeding of *A. alosa* with enriched *Artemia* nauplii or co-feeding *T. aceti* might not only hamper the development of cranial malformations under captive rearing conditions, but could also decrease mortality upon release.

To our knowledge nobody demonstrated the effect of water hardness on mortality during OTC/TC marking so far in a freshwater environment. Our research regarding the possible effect of Ca^{2+}- and Mg^{2+}-ions on Tetracycline marking (Chapter IV) highlights the fact, that water hardness is a consistently underestimated factor that should be taken into consideration when developing marking protocols. Within the framework of the LIFE-project the transfer of marking protocols established in Saint-Seurin sur l'Isle (France) to the Eußerthal rearing station in Germany resulted in the mortality of approximately 6000 shads (> 50% mortality). As the protocol was closely followed and the abiotic factors pH, dissolved oxygen and temperature were continuously monitored the observed rise in mortality can most likely be explained by the low water hardness of ~5.5°dH found in the Eußerthal research facility, which correspondingly lead to elevated mortality during TC-marking experiments with zebra-fish (Chapter IV). Also the absence of TC-marks that has been observed in a number of shad specimens being marked in the Allis shad hatchery of Bruch despite closely following the marking protocol (personal observation Matthias Hundt), might have to be attributed to the effect of TCcomplexation. As the concentration of Ca^{2+}- and Mg^{2+}-ions in Garonne River does show considerable short term variation (Etchangu & Probst 1988) water hardness in the LIFE-hatchery of Bruch, which is fed with water derived from Garonne River, is hardly predictable and adaptation of the marking protocol to changing conditions is difficult. Therefore low marking success can potentially be caused by elevated water hardness, as observed in sagittal otolith marks of Palmetto Bass being marked at 500 mg $CaCO_3$ (~28 °dH) (Mauk 2008). Despite being one of the most widely applied chemicals in stock enhancement and restocking programmes, effects on a sub-lethal level of TC-derivates have so far not been evaluated and most investigation are exclusively focusing on direct mortality during and shortly after marking e.g. Secor, White & Dean (1991) and Denson & Smith (2008). Meyer *et al.* (2012) demonstrated that marking of cod eggs and yolk-sac larvae with alizarin complexone can effect several sublethal parameters as for example first feeding success and growth during exogenous feeding. Since TC-derivates represent one of the most frequently used antibacterial agents for fish (Lunestad & Goksøyr 1990), sublethal effects potentially impairing larval survival after release seem very likely. In light of the numerous problems connected to the marking quality (Chapter VII) and potential hazards (Chapter IV) of TC-immersion marking with larvae of *A. alosa,* I would like to advocate the use microsatellite based multiloci parentage analysis as an alternative identification method in order to discriminate wild from hatchery-reared individuals. Parentage analysis has been successfully applied in the restoration of populations of the Atlantic salmon in Connecticut River (Letcher & King 2001) and has served as a valuable tool in identifying hatchery-reared conspecifics of several other species (e.g. Jeong *et al.* 2007; Fisch *et al.* 2012). Furthermore it offers several advantages in comparison to chemical marking techniques: it is a non lethal identification method, as sampling can be performed by removing small amounts of tissue such as scales (May, Krueger & Kincaid 1997). Parentage analysis can be used as a tool in evaluating the performance of captive breeding efforts and thereby minimize average co-ancestry and limit inbreeding (Fisch *et al.* 2012). In order identify possible outbreeding effects of stocking fry from the adjacent Pamunkey River population in James River microsatellite data has been used during restoration efforts of the American shad *A. sapidissima* (Virginia, USA).

Implications for the *conservation and restoration* of populations of Allis shad in River Rhine and throughout Europe

The observation of natural reproduction in 2013 after five years of stocking (Chapter VII), as well as the apparent rise in number of upstream migrating individuals in 2014 (Chapter VII) are clear indicators for the success of the reintroduction programme for Allis shad in River Rhine. The occurrence of natural reproduction was demonstrated for all three fish species so far re-introduced into river Rhine, shortly after the initial stocking (Molls & Nemitz 2008; Borcherding *et al.* 2010; Borcherding 2011). However, whilst North Sea houting (*Coregonus spp.*) successfully established a self-sustaining population within less than 10 years, the Atlantic salmon (*S. salar*) is after 26 years of stocking several million individuals still strongly dependent on supportive stocking measures (Molls & Nemitz 2008; Borcherding *et al.* 2010; Borcherding 2011). The biggest problem with regard to the re-establishment of Atlantic salmon populations is considered the inadequate quality of available spawning grounds, which rarely provide sufficient levels of oxygen to allow the development of eggs, due to eutrophication effects as well as altered flow conditions and the consequential clogging of the interstitial system (Molls & Nemitz 2008). In contrast to salmon Allis shad and North Sea houting do not require the presence of well-oxygenized gravel beds. Although displaying different preferences in choice of their specific spawning habitat (Aprahamian *et al.* 2003; Borcherding, Scharbert & Urbatzka 2006), both of these species freely spawn into the water column above substrate of various sizes at moderate water velocities and therefore large stretches of the main channel of river Rhine are available as spawning habitat. For Allis shad 65 suitable spawning grounds were identified downstream of the lock of Iffezheim (Klinger 2011) in the main channel of River Rhine. As monitoring data of the fish passes of Gambsheim and Iffezheim demonstrate shad are spawning even further upstream (Chapter VII). Furthermore Allis shad have a considerably higher specific fecundity than Atlantic salmon and also when compared to North Sea houting (Jäger 1999; Bagliniere *et al.* 2003; Jonsson & Jonsson 2011). Therefore the re-introduction of *A. alosa* in River Rhine does not seem to be limited by the same obstacles as in the case of Atlantic salmon.

The man-made introduction of the American shad *A. sapidissima* into several pacific rivers of North America where this species is not indigenous, gives an clear indication of the tremendous colonization potential of alosine species. The stocking of few million shad larvae to Columbia River and adjacent river systems (a total of 910 000 larvae was stocked directly to Columbia River) in the 19th century lead to the single largest spawning run of this river system with over 4 million annual returners within the first decade of the 21st century (Hasselman *et al.* 2012). The remarkable invasion of Columbia River has been largely explained with *A. sapidissimas* high specific fecundity, which is very similar to *A. alosa and* the access to vast areas of suitable spawning habitat (Bagliniere *et al.* 2003; Hasselman *et al.* 2012). Additional to these factors the warmer temperature of Columbia River after the gradual conversion of the original river basin, favoured American shad by expanding its niche breadth and providing conditions for increased reproductive success (Hasselman *et al.* 2012). According to our investigations the temperature tolerance of Allis shad surpasses that of the Atlantic salmon, the North Sea houting and most other native fish species of River Rhine (Chapter VI and Leuven *et al.* (2011)). In light of the constantly rising water temperatures of River Rhine (Leuven *et al.* 2007) the high temperature tolerance of larval *A. alosa* (Chapter VI) can therefore be considered a further characteristic in favour of a successful recolonisation of this species in the long-term prospect.

Although substantial effort has been invested in the re-establishment of linear passability of River Rhine, the successful upstream migration of shad is still hampered by several obstacles. Particularly the storm surge barrier “Haringvliet” situated in the Rhine-Maas-delta can be considered a major migration barrier for shad, as it cannot only prevent mature shad from entering River Rhine, in order to start their upstream spawning migration, but may also interfere downstream-migration by altering the brackish water environment of the Rhine Delta to freshwater conditions (Brenner *et al.* 2004; Klinger 2011). Secondly the access to several of the largest Rhine tributaries is limited due to the absence of suitable fish-passes or the inadequacy of certain fish pass designs to support the upstream migration of shad (Klinger 2011). The fact that since the beginning of monitoring operations in the fish-pass of Gambsheim, every year more upstream migrating shad were registered in Gambsheim compared to the downstream located Iffezheim monitoring station, is a strong indication for the inadequate fish-pass design of Iffezheim with regard to Allis shad. The design of fish-passes with a high efficiency to transport Allis shad is difficult and in many cases it has been shown that adjacent locks often provide a better transport possibility than the original fish pass (Travade & Larinier 2002). Therefore particular attention should be paid to the specific migratory requirements of shad when constructing future fish passes for this species. Besides the promising initial steps towards the successful re-introduction of *A. alosa* in River Rhine, the collapse of Allis shad stocks in the Gironde watershed (Rougier *et al.* 2012) reveal that population dynamics of this species are so far hardly understood. Therefore future investigations regarding the larval and juvenile ecology of *A. alosa*, as well as research on adult mortality at sea are necessary in order to understand the underlying mechanisms of recruitment for this species.

As shown in this thesis the successful re-introduction of Allis shad is dependent on a variety of factors, ranging from the handling of larvae during the production process to physio/chemical factors influencing migratory pathways and habitats of this species throughout its lifecycle. However by the successful introduction of Allis shad in River Rhine with wild shad from River Garonne, I clearly illustrated that the recolonisation of major river systems within *A. alosas* historical distribution range by artificial propagation is possible and has to be considered a viable conservation strategy.

Summary

The largest population of the anadromous Allis shad (*A. alosa)* of the 19th century was found in River Rhine and has to be considered extinct today. To facilitate the return of *A. alosa* into River Rhine an EU LIFE-project was initiated in 2007. The overall objective of this thesis was to assist *aquaculture and stocking*-measures at River Rhine, as well as to support *restoration and conservation* of populations of Allis shad in Europe.

By culturing the free-swimming nematode *T. aceti* in a solution of cider vinegar we developed a cost-effective live food organism for the larviculture of fish. As indicated by experiments with *C. maraena, T. aceti* cannot be regarded as an alternative to *Artemia* nauplii. However it has to be considered a suitable supplemental feed in the early rearing of *C. maraena* by providing essential fatty acids, thereby optimizing growth.

Also mass-marking practices with Oxytetracycline, as they are applied in the restocking of Allis shad have been evaluated. In experiments with *D. rerio* we demonstrated that water hardness can detrimentally affect mortality during marking and has to be considered crucial in the development of marking protocols for freshwater fish.

In order to get independent from wild spawners an ex-situ Broodstock-facility for Allis shad was established in 2011. Upon examination of two complete year classes of this broodstock, we found a high prevalence of various malformations, which could be traced back to distinct cysts developing one month post hatch. Despite applying a variety of clinical tests we could not identify any infectious agents causing these malformations. The observed malformations are probably a consequence of suboptimal feeding practices or the properties of the physio-chemical rearing environment.

The decline of stocks of *A. alosa* in Europe has been largely explained with the increase of river temperatures as a consequence of global warming. By investigating the temperature physiology of larval Allis shad we demonstrated that *A. alosa* ranges among the most thermo-tolerant species in Europe and that correlations between rising temperatures and the disappearance of this species have to be understood in a synecological context and by integrating a variety of stressors other than temperature.

By capturing and examining juvenile and adult Allis shad from River Rhine, we demonstrated the first natural reproduction of *A. alosa* in River Rhine since nearly 100 years and the success of stocking measures within the framework of the LIFE project.

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Appendix

A.1 Measures for the preparation of the re-introduction of *A. alosa* based on IUCN requirements in preparation of the LIFE project:"The re-introduction of Allis shad (*A. alosa*) in the Rhine System"

Selection of IUCN prerequisites modified by (IUCN 1998)	Pre-project action (Beeck 2004)
Detailed studies should be made of the status and biology of wild populations (if they exist) to determine the species' critical needs.	A detailed literature study on all aspects of A. *alosas* biology was performed and initial investigations regarding a possible re-introduction were performed.
Availability of suitable habitat: re-introductions should only take place where the habitat and landscape requirements of the species are satisfied, and likely to be sustained for the for-seeable future. The area should have sufficient carrying capacity to sustain growth of the re-introduced population and support a viable (self-sustaining) population in the long run.	Suitable spawning habitats were identified in North Rhine-Westphalia based on observations of current French spawning habitats.
Identification and elimination, or reduction to a sufficient level, of previous causes of decline.	River pollution and fisheries are not critical factors for Allis shad nowadays, while the linear passability of major tributaries is still impaired. Nevertheless the causes of decline are considered to be sufficiently reduced, to allow recolonisation of River Rhine.

It is desirable that source animals come from wild populations. If there is a choice of wild populations to supply founder stock for translocation, the source population should ideally be closely related genetically to the original native stock and show similar ecological characteristics. Removal of individuals for re-introduction must not endanger the captive stock population or the wild source population.	Single individuals caught in River Rhine were compared to extant living populations of *A. alosa.* No genetical evidence for a residual population in River Rhine was found. Due to the high genetic diversity and the fact that all strays found in River Rhine are originating from River Garonne. The Garonne shad were chosen as the donor population of River Rhine.
Post release monitoring is required of all (or sample of) individuals. This most vital aspect may be by direct (e.g. tagging, telemetry) or indirect (e.g. spoor, informants) methods as suitable	A suitable chemical marking method was developed to identify stocked individuals.

Record of achievement

Chapter II: I extensively contributed to the data analysis and manuscript production.

Chapter III: I extensively contributed to the study-design and laboratory work. Data analysis and manuscript production is exclusively based on my work, contribution of the co- authors is mainly based on improvements and amendment statements with regard to content.

Chapter IV: Study design, laboratory work, data analysis and manuscript production is exclusively based on the work of Benjamin Schreiber and me (these authors contributed equally). Contribution of other coauthors is mainly based on improvements and amendment statements with regard to content.

Chapter V: I contributed to study design, laboratory work, data analysis and manuscript production.

Chapter VI: Study design, data analysis and manuscript production is exclusively based on my work. Other coauthors participated in laboratory work and helped with improvements and amendment statements with regard to content.

Chapter VII: Study design, data analysis and manuscript production is exclusively based on my work. Other coauthors participated in laboratory work and helped with improvements and amendment statements with regard to content

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