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**Master of Science (Biochemistry)**

**Establishing a system to study the evolution of**  
**copper resistance in the great duckweed**  
**(*Spirodela polyrhiza*)**

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## 1 Abstract

Due to an increase of environmental pollution with heavy metals such as lead or copper plants need to deal with these stress factors. One mechanism to reduce heavy metal stress is the production of metal chelators and antioxidants such as flavonoids. Although flavonoids may be involved in heavy metal resistance the evolutionary response of these compounds to heavy metal exposure remains little understood. In order to shed light into these processes, three lines of experiments were set up in the aquatic great duckweed *Spirodela polyrhiza*.

First it was tested whether copper resistance is associated with flavonoid concentrations across 53 natural *S. polyrhiza* genotypes. Second, it was analyzed whether exposure of copper affects the offspring's ability to cope with this stress factor. Third, monoclonal *S. polyrhiza* populations were experimentally evolved for 40 generations on copper and tested for heavy metal resistance and flavonoid concentrations.

The variation in flavonoid concentration of 53 natural *Spirodela polyrhiza* genotypes is positively correlated with plant resistance under copper stress. Offspring of copper-challenged plants had higher plant fitness under control conditions but lower fitness under copper stress than offspring of non-challenged plants. Parental exposure to copper also affected flavonoid concentrations in the offspring: under copper stress, offspring of copper exposed plants induced the major flavonoids luteolin-7-O-glucoside and luteolin-8-C-glucoside stronger than offspring of unexposed plants. To a small extent offspring of copper-exposed and non-exposed plants also induced apigenin-7-O-glucoside and apigenin-8-C-glucoside under copper stress. In contrast, *S. polyrhiza* plants that were evolved for 40 generations on copper lacked the increased growth response under control conditions and failed to induce flavonoids more than control-evolved plants, indicating that evolution on copper eliminated these responses. Furthermore, although copper and control-evolved plants had similar overall plant fitness under copper stress, copper-evolved plants performed worse than control-evolved plants for the first eight days of growth, a pattern that was reversed in the subsequent eight days.

Taken together, these experiments provide first evidence that flavonoids might be involved in copper resistance in *S. polyrhiza*, and highlight the potential of multi-generational experiments to elucidate plant evolution to environmental stresses.

## 2 Zusammenfassung

Aufgrund steigender Umweltverschmutzung durch Schwermetalle wie Blei oder Kupfer sind Pflanzen gezwungen mit diesen Stressfaktoren umzugehen. Ein Mechanismus um Schwermetall-Stress zu reduzieren ist die Produktion von Metall-komplexierenden und anti-oxidativen Verbindungen wie Flavonoiden. Über die Evolution dieser Verbindungen auf Schwermetall-Exposition ist wenig bekannt. Um Einsicht in diese Prozesse zu erlangen wurden drei Experimente mit der Wasserlinse *Spirodela polyrhiza* durchgeführt. Zunächst wurde in 53 natürlich vorkommenden *S. polyrhiza* Genotypen überprüft, ob ein Zusammenhang zwischen Kupferresistenz und Flavonoidkonzentration besteht. Weiterhin wurde analysiert, ob Kupferexposition die Fähigkeit der Nachkommen mit diesem Stressfaktor umzugehen beeinflusst. Drittens wurden monoklonale *S. polyrhiza* Populationen für 40 Generationen experimentell unter Kupferexposition evolviert und anschließend Schwermetallresistenz und Flavonoidkonzentrationen überprüft.

Die Variation der Flavonoidkonzentrationen in 53 *S. polyrhiza* Genotypen ist positiv mit der Resistenz der Pflanzen gegenüber Kupferstress korreliert. Nachkommen kupfer-exponierter Pflanzen zeigten unter Kupferstress eine erhöhte Fitness unter Kontrollbedingungen, jedoch eine geringere Fitness als Kontroll-Pflanzen. Elterliche Kupferexposition beeinflusste auch die Konzentrationen der vier Hauptflavonoide in den Nachkommen: unter Kupferstress akkumulierten Nachkommen von kupferexponierten Pflanzen Luteolin-7-O- und -8-C-Glucosid stärker als Nachkommen nicht-exponierter Pflanzen. In geringem Maß akkumulierten unter Kupferstress auch Apigenin-7-O- und -8-C-Glucosid in Nachkommen kupferexponierter Pflanzen und Kontroll-Pflanzen. Im Gegensatz dazu zeigten *S. polyrhiza* Pflanzen die für 40 Generationen unter Kupferexposition wuchsen kein erhöhtes Wachstum unter Kontrollbedingungen und sie akkumulierten nicht mehr Flavonoide als Kontrollpflanzen. Dies deutet darauf hin, dass die Kultivierung unter Kupferstress diese Phänomene eliminierte. Trotz gleicher Gesamtfitness der Pflanzen, die unter Kupfer- und Kontrollbedingungen evolviert wurden, wuchsen in den ersten acht Tagen kupferevolvierte Pflanzen schlechter als Kontrollpflanzen. In den darauffolgenden acht Tagen kehrte sich dies um.

Zusammenfassend liefern diese Experimente erste Hinweise darauf, dass Flavonoide an der Kupferresistenz von *S. polyrhiza* beteiligt sein könnten und zeigen das Potential multi-generationeller Experimente um pflanzliche Anpassung an Umweltstressoren zu untersuchen.

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**III. List of Abbreviations**

Ø	diameter
ANOVA	analysis of variance
Apig7O	apigenin-7-O-glucoside
Apig8C	apigenin-8-C-glucoside
CHI	chalcone isomerase
CHS	chalcone synthase
CoA	coenzyme A
Cu/ZnSOD	copper/zinc superoxide dismutase
DAD	diode array detector
DNA	deoxyribonucleic acid
dpt	days post transplantation
FW	fresh weight
glc	glucose
HPLC	high-performance liquid chromatography
ln	natural logarithm
Lut7O	luteolin-7-O-glucoside
Lut8C	luteolin-8-C-glucoside
Me <sup>2+</sup>	metal ions
N-medium	full nutrient medium
nr	number
PAL	phenylalanine ammonia lyase
Po	pouch
RNA	ribonucleic acid
ROS	reactive oxygen species
Sti	stipe
TA-G	taraxinic acid $\beta$ -D-glucopyranosyl ester
UV	ultra violet

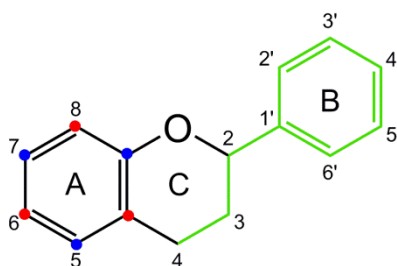
### 3 Introduction

Due to their sessile life style plants have to adapt consistently to changing environmental conditions including various biotic and abiotic factors such as pathogenic fungi and insect herbivores, as well as light irradiation, nutrient and water availability.

As a protection against such stress factors plants have developed mechanical barriers like thorns against herbivores or the cuticle as a protection against water evaporation, as well as a variety of chemical compounds [1]. These so-called secondary metabolites are chemicals that are not necessary for growth and development of the plant but are used as a protection against adverse environmental conditions. They are, for example, beneficial under certain conditions like insect herbivory or UV stress. Unlike the primary metabolism, which includes all reactions that are essential for the life processes of a cell, the secondary metabolism is about chemical specialization of plant species in order to adapt to their environment [2]. For example, the sesquiterpene lactone TA-G (taraxinic acid  $\beta$ -D-glucopyranosyl ester) is found in *Taraxacum officinale* latex and has deterrent effects on *Melolontha melolontha* larvae, and thus protects the roots [3]. Secondary metabolites belong to chemically diverse groups such as terpenoids, alkaloids, glucosinolates and phenolic compounds [4]. One of the largest, most ancient and diverse group of chemicals are flavonoids, which are phenolic compounds.

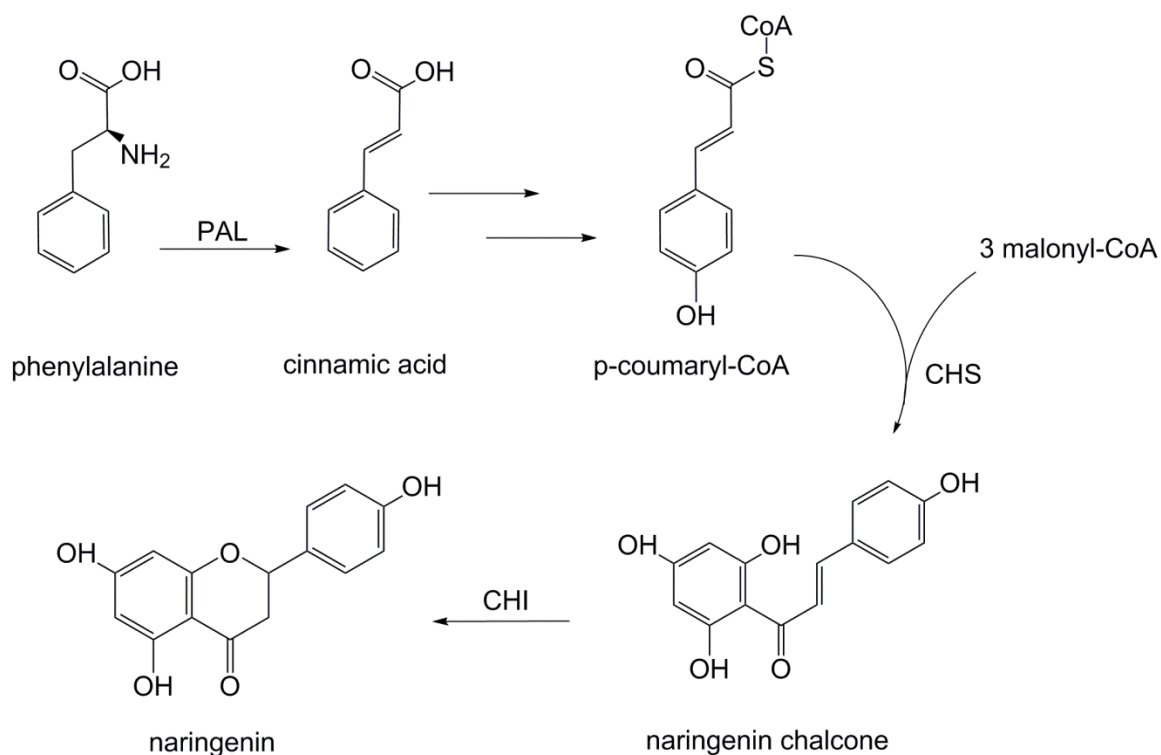
#### 3.1 Flavonoids

Flavonoids are a group of secondary metabolites with a benzo- $\gamma$ -pyrone-based structure [5]. The synthesis of the basic fifteen-carbon skeleton (Figure 3-1) involves the shikimic acid pathway and the polyketide pathway [2].



**Figure 3-1: Basic flavonoid structure.** Two benzene rings (A and B) are linked via heterocyclic pyrane ring (C). The Carbon atoms derive from the shikimic acid pathway (green) and the polyketide unit from acetate units ( $\text{CH}_3$  red –  $\text{COOH}$  blue). Modified according to [1].

Flavonoids are synthesized through the phenylpropanoid pathway (Figure 3-2). Phenylalanine is transformed to cinnamic acid by the enzyme PAL (phenylalanine ammonia lyase) in the first step of this pathway [6]. The first enzyme specific for the subsequent flavonoid pathway catalyzes the condensation of p-coumaryl-CoA with three malonyl-CoA molecules [6]. Thereby the so-called chalcone synthase (CHS) produces a chalcone scaffold with an open C-ring from which all flavonoids are derived [6]. In the next step the chalcone is converted to naringenin, a key substrate for different enzymes and therefore a branching point in the pathway [7].



**Figure 3-2: Phenylpropanoid pathway and biosynthesis of the flavanone naringenin.** PAL = phenylalanine ammonia lyase; CHS = chalcone synthase; CHI = chalcone isomerase; CoA = coenzyme A.

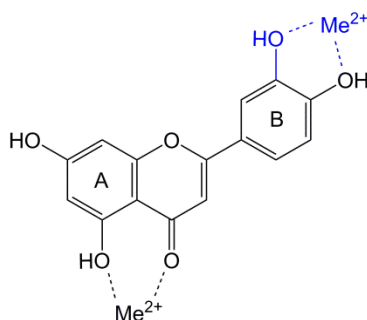
According to the level of oxidation and the substitution pattern of the C-ring the flavonoids are divided into subgroups. Flavones for instance contain a carbonyl group on C4 and a C-C double bond between C2-C3 (e.g. luteolin, apigenin). Flavonols have also a carbonyl group on C4 and a C-C double bond between C2-C3 and in addition a hydroxyl group C3 (e.g. kaempferol), whereas flavanones contain only the carbonyl

substitution on C4 (e.g. naringenin) [5]. Within these classes the compounds differ in the substitution pattern of the two benzene rings (A- and B-ring in Figure 3-1).

Furthermore, flavonoids may occur as aglycones, glycosides and methylated derivatives [5]. These modifications influence the chemical characteristics of the flavonoids. Glycosylation is a widespread modification of secondary metabolites catalyzed by glycosyltransferases [8]. It increases water solubility as well as stability through protection of reactive nucleophilic groups and protection from auto-oxidation [5, 8]. Also, flavones like apigenin and luteolin are often O- and C-glycosylated (Figure 3-5), especially on their most reactive hydroxyl group, which is 7-OH [5].

Flavonoids have diverse biological functions [6]. They are involved in transporting the phytohormone auxin, and protecting against phytopathogens [6]. Flavonoids are also useful in protecting against abiotic stressors such as UV radiation [6], and are potent scavengers of reactive oxygen species (ROS), thereby preventing peroxidation of lipids [9]. Furthermore flavonoids seem to play a role in heavy metal stress tolerance. In *Arabidopsis thaliana* seedlings heavy metal stress decreased growth of mutants with defects in the flavonoid biosynthesis [10]. Adding the flavonoids naringenin and quercetin partially restored the growth of the mutants under cadmium stress [10]. In safflower (*Carthamus tinctorius*) flavonoids are significantly increased in leaves of copper stressed seedlings [11] and in grapevine PAL and chalcone synthase, enzymes of the phenylpropanoid pathway, are up-regulated under copper stress [12].

It is possible that flavonoids benefit plant performance under heavy metal stress by scavenging of ROS as well as by complexing metal ions. Thereby *ortho*-dihydroxylated B-ring flavonoids have a higher potential to bind trace metal ions such as copper ions than their monohydroxylated counterparts [13].



**Figure 3-3: Binding sites for trace metals in the flavonoids apigenin and luteolin.** Apigenin has one binding site for metal ions (black), whereas luteolin has an additional binding site due to the 3' hydroxyl substitution (blue).  $\text{Me}^{2+}$  indicates metal ions. Modified according to [5].

### 3.2 Impact of Copper Stress on Plants and Mechanisms of Plant Adaption

Copper is an essential trace metal that is required by plants for normal growth and development [14]. It is necessary as a cofactor in enzymes such as copper/zinc superoxide dismutase (Cu/ZnSOD), ascorbate oxidase or cytochrome c oxidase [14]. Under physiological conditions copper occurs as cupric ( $\text{Cu}^+$ ) and cuprous ( $\text{Cu}^{2+}$ ) ions [14].

Due to high anthropogenic emission of heavy metals such as copper during mining processes or use of fertilizers in agriculture, the environment is polluted with this heavy metal [15].

Excess copper severely impacts on plant development [15]. Affected plants show root and shoot growth retardation/inhibition, chlorosis and necrosis [14]. The toxicity of copper may have many reasons. First, the enzyme activity could be inhibited by copper-ions, especially  $\text{Cu}^+$ , interacting with the sulphur of the amino acids cysteine or methionine in proteins [14]. Furthermore, copper may lead to deficiencies of other essential ions and displacement of essential cations from specific binding sites; e.g. the substitution of the central  $\text{Mg}^{2+}$  ion of chlorophyll by  $\text{Cu}^{2+}$  leads to inhibition of photosynthesis [14]. Also, the cell transport processes are impaired. Last but not least,  $\text{Cu}^+$  can form hydroxyl radicals by the disproportionation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) via Haber-Weiss cycle [15]. The formation of reactive oxygen species (ROS) is a major mechanism of copper toxicity [14].

#### Mechanisms of Adaption

Elucidating the mechanisms with which organisms adapt to the environment has been a major goal in ecology and evolution. Most of the evolutionary theories and hypotheses are tested by studying patterns, e.g. divergence between species and populations. All these studies reflect past events and thus it often remains difficult to infer the selective agent as well as to rule out confounding effects, e.g. due to underlying population structure. In the last decades, experimental evolutionary studies have been increasingly applied to study adaptation in real time, which allows controlling both the selective regime as well as the founder gene pool. As these experiments require fast generation time and large population size, experimental evolution studies on higher plants remain scarce, which constrains our understanding of the adaptation process in these organisms.

Since modern evolutionary synthesis, phenotypic differences are thought to originate from genetic variation among individuals of a population [16]. However, it has become

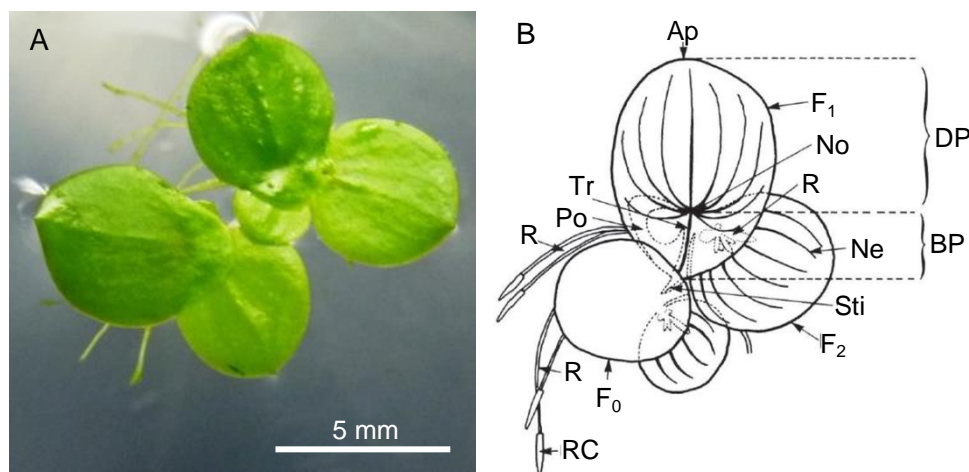
increasingly clear during the last two decades, that phenotypes are not only due to genetic but also epigenetic (defined here as mitotically and/or meiotically heritable changes in gene expression that occur without a change in DNA sequence [17]) variation. For example, a natural occurring mutant of toadflax (*Linaria vulgaris*), has radially instead of bilaterally symmetric flowers [18]. This phenotype is stably transmitted and is caused by an extensive methylation of the *cycloidea* (*cyc*) gene, which is therefore transcriptionally silent [18]. Several biochemical mechanisms may account for such epigenetic effects, including methylation of the DNA, histone modification and small RNAs [19, 20].

Epigenetic variation may arise randomly similar to genetic mutations [21, 22], albeit at about 1000 fold higher frequency, or they may be induced by the environment [23, 24]. Such environment-induced epigenetic variation, if they lead to an adaptive phenotype and are transmitted to offspring, offer the possibility of rapid evolutionary changes. Such mechanisms may be especially important in clonal organisms, where the lack of recombination limits the accumulation of beneficial alleles. However, it has remained difficult to demonstrate environment-induced adaptive transgenerational epigenetic effects in plants, partially because these organisms may affect offspring phenotype not only by inheriting a specific genetic and epigenetic makeup, but for example also through nutrient provisioning or contribution of bioactive molecules in seeds [25]. Many of these so called parental effects do not have an epigenetic basis and will not be inherited over multiple generations. Thus, in order to infer an epigenetic basis of transgenerational inherited traits, the effects must be inherited over multiple generations. Furthermore, genetic effects on the phenotype must be ruled out. Thereto, clonal organisms that completely lack genetic variation offer great opportunity to study transgenerational epigenetic effects.

### 3.3 *Spirodela polyrhiza* as Model Organism

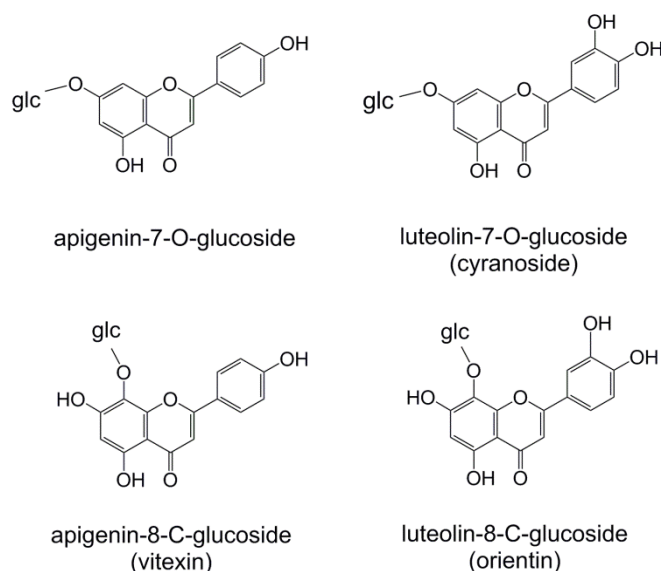
Duckweeds (Lemnoidea, Araceae) are the world's smallest and fastest growing angiosperms. They are divided into five genera (Landoltia, Lemna, Spirodela, Wolffia and Wolffia) and contain 38 species [26, 27]. The aquatic plants grow in stagnant and slowly flowing waters [28]. The body plan of duckweeds is highly reduced, consisting of a leaf-like thallus, so called frond, and depending on the species zero to several roots [28]. Duckweeds are attractive model organisms in terms of biofuel production and phytoremediation, for example for waste water clearance [29].

The common duckweed *Spirodela polyrhiza* is with its approximately 5 to 10 mm in diameter large fronds the largest species of the Lemnoideae subfamily [28]. *Spirodela polyrhiza* reproduces almost exclusively by asexual budding with duplication rates of 2-3 days under optimal growth conditions [28]. As shown in Figure 3-4, the daughter fronds ( $F_1$ ) derive from two lateral meristematic pockets (Po) of the mother frond ( $F_0$ ), with a stipe (Sti) connecting the fronds [30]. Importantly, fronds are initiated at a very early stage during development - thus, an emerging frond can already contain three or more daughter fronds [28].



**Figure 3-4: Vegetative growth of *Spirodela polyrhiza* fronds by asexual budding.** (A) Group of fronds of strain 7498 shown in plan view. (B) Schematic drawing of a group of fronds during budding, Ap = apex; BP = basal part of the frond; DP = distal part of the frond;  $F_0$  = mother frond;  $F_1$  = daughter frond of the first generation;  $F_2$  = daughter frond of the second generation; No = node; Po = pouch; R = root; RC = rootcap; Sti = stipe connecting mother and daughter frond; Tr = tract of elongated cells connecting stipe and node; modified according to [28].

The genome of *Spirodela polyrhiza* is one of the smallest amongst plants (158 Mbp, similar to the genome of *Arabidopsis thaliana*) and has recently been sequenced for the strain 7498 [31].



**Figure 3-5: The four major flavonoids of *Spirodela polyrhiza*.** Common synonyms are indicated in brackets. glc = glucose.

*Spirodela polyrhiza* is rich in flavonoids as well as anthocyanins. Among a total of 18 identified flavonoids, the four flavones luteolin-7-O-glucoside, luteolin-8-C-glucoside, apigenin-7-O-glucoside and apigenin-8-C-glucoside are predominant [32]. Their chemical structures are shown in Figure 3-5. Apigenin (phenol) and luteolin (catechol) are structurally different only in one hydroxyl group of the B-ring [33] leading to a potential difference for oxidative stress resistance. The *ortho*-3',4'-dihydroxy substitution in the B-ring of luteolin is important for  $\text{Cu}^{2+}$ -chelate formation thus has a much higher antioxidative potential than monohydroxylated flavonoids [13].

In summary, the short generation time and small plant size, the availability of a high-quality reference genome, the well characterized secondary chemistry provide a unique opportunity to study the evolution of secondary metabolites in response to different stresses.

### 3.4 Aim of Study

The aim of this study is to assess the evolution of copper resistance in *Spirodela polyrhiza* with particular focus on the role of flavonoids in the adaption process.

The study contains three major projects:

First, investigate whether *Spirodela polyrhiza* genotypes vary in their resistance to copper and whether variation in flavonoid concentration correlates with plant resistance under copper stress.

Second, it was tested whether exposure to copper affects plant fitness and flavonoid levels of subsequent generations under the same stress condition.

Third, large populations of monoclonal *Spirodela polyrhiza* populations were evolved for approximately 40 generations on copper and subsequently analyzed plant fitness and flavonoid levels of evolved and non-evolved populations in the presence and absence of copper stress.

## 4 Materials and Methods

### 4.1 Materials

Chemicals were supplied by Roth GmbH and Co. KG (Karlsruhe, Germany) and Sigma Aldrich (Steinheim, Germany) in analytical grade quality, if not noted otherwise.  $\text{CuSO}_4$  solutions were prepared with deionized and filtered water acquired from a Milli-Q integral water purification system (pore size: 0.22  $\mu\text{m}$ , Merck Millipore, Darmstadt, Germany). N-medium (Table 4-2) was prepared with deionized water. Solvents used for HPLC-UV/Vis were supplied by VWR PROLABO BDH chemicals (Leuven, Germany) and Fisher Chemical (Thermo Fisher Scientific, Waltham, USA) in analytical grade quality. Luteolin-7-O-glucoside was obtained from Extrasynthese (Genay, France).

#### 4.1.1 Equipment

A detailed list of the used equipment is shown below in Table 4-1.

**Table 4-1: Equipment**

Item	Specifications	Producer
Balance	BP310S	Sartorius (Göttingen, Germany)
Centrifuge	Heraeus Pico17	Thermo Fisher Scientific (Waltham, USA)
Climate Chamber	Growth chamber	York Refrigeration (York, USA)
Column Nucleodur Sphinx RP	250 x 4.6 mm, 5 $\mu\text{m}$ particle size	Macherey-Nagel (Düren, Germany)
Filter	Vorfilter 25 cm	Einhell ISC GmbH (Landau/Isar)
Fine Balance	BP211D	Sartorius (Göttingen, Germany)
HPLC-UV/Vis	Series 1100; Agilent	Hewlett-Packard/ Agilent Technologies (Santa Clara, USA)
Light sources	Osram Lumilux L36 W/ 865 cool daylight	Osram GmbH (Munich, Germany)
Milli-Q (0.22 $\mu\text{m}$ filter)	Synthesis A10	Merck Millipore (Darmstadt, Germany)
Mixer	Vortex Genie 2 <sup>TM</sup>	Scientific Industries (Bohemia, USA)
Photodiode array detector	G1315A DAD	Agilent Technologies (Santa Clara, USA)

Item	Specifications	Producer
Shaker (ball mill)	Skandex SO-10m	Fast & Fluid (Sassenheim, Netherlands)
Water pump	Aquarium pump Eden 155	PfG GmbH (Hörstel, Germany)

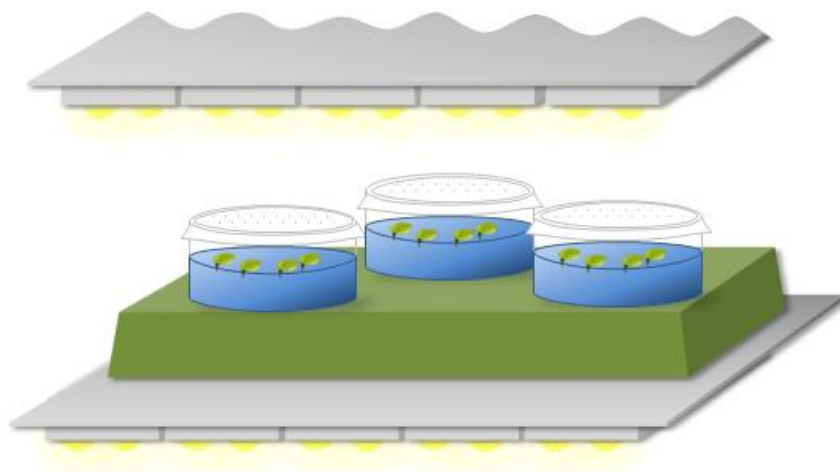
#### 4.1.2 Software

For acquisition and analysis of the obtained data the following software programs were used: Agilent ChemStation Rev. A.09.01[1206], DataTrans, Microsoft Office 2010 and R (Version 3.3.1). Graphical illustration was performed with Adobe Illustrator CS5 (64bit) and ChemDraw Professional (Version 15.1.0.144). Furthermore EndNote X7.7 was used.

## 4.2 Methods

### 4.2.1 Plant Cultivation

All experiments were performed with *Spirodela polyrhiza* plants. For transgenerational inheritance and evolution experiments genotype 7498 USA was used. The plants were cultivated in a growth chamber at 26 °C in long-day conditions (16:8 hours light:dark) with a light intensity of 130 to 160  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 40% humidity. Plastic beakers, boxes and tubes (see supplement Table 10-1) were set on trays of 6 cm height to prevent overheating of the medium resulting from the subjacent light sources. A schematic drawing is shown in Figure 4-1. To support fast reproduction plants were grown in N-medium (Table 4-2).



**Figure 4-1: Schematic drawing of duckweed cultivation in plastic beakers.** Plastic beakers with perforated lids as well as boxes and tubes were set on trays to prevent overheating of the medium resulting from the subjacent light sources.

**Table 4-2: Composition of N-medium (full nutrient medium)**

Compound	Concentration
KH <sub>2</sub> PO <sub>4</sub>	150 µM
Ca(NO <sub>3</sub> ) <sub>2</sub>	1 mM
KNO <sub>3</sub>	8 mM
H <sub>3</sub> BO <sub>3</sub>	5 µM
MnCl <sub>2</sub>	13 µM
Na <sub>2</sub> MoO <sub>4</sub>	0.4 µM
MgSO <sub>4</sub>	1 mM
FeNaEDTA	25 µM

#### 4.2.2 Genotype Resistance

In order to test whether flavonoid production correlates to plant fitness under copper stress, 53 *Spirodela polyrhiza* genotypes were grown the presence and absence of 10 µM copper. The *S. polyrhiza* genotypes (see supplement Table 10-2) were obtained from Dr. K. Appenroth and selected based on their geographic dispersed origin. The plants were precultivated for 1 week in Erlenmeyer flasks (26°C, 16:8 hours light:dark) in N-medium (Table 4-2). For each genotype five mature fronds with small daughter fronds were placed into 250 mL plastic beakers filled with 150 mL N-medium with (n = 3) and without (n = 3) 10 µM CuSO<sub>4</sub>. The beakers were covered with transparent and perforated plastic lids and a white fleece (Agrarvlies, 17 g/m<sup>2</sup>). The plants were incubated for seven days in a growth chamber (26°C constant, 16:8 hours light:dark, 160 µmol m<sup>-2</sup> s<sup>-1</sup>). After seven days the number of green and yellow/brown fronds (criteria for brown fronds: 50% or more of frond surface brown) was counted and the total fresh weight was determined. Samples of around 50 mg were weighted, immediately frozen in liquid nitrogen in Eppendorf tubes and stored at -20°C until further analysis.

#### 4.2.3 Transgenerational Inheritance

To investigate whether exposure to copper in parental generations benefits the offspring under copper stress, *S. polyrhiza* plants were cultivated in transparent plastic boxes with 4x10 wells. In these 40-hole racks five generations of *S. polyrhiza* grew in 40 mL N-medium with or without 20 µM CuSO<sub>4</sub>, with 20 plants per treatment ("background

treatment”). The boxes were covered with transparent plastic lids with a space of 1 mm to the box for air flow.

Since plants in control medium grow faster compared to plants in CuSO<sub>4</sub>-containing medium, the propagation of the control group was started with five days delay. Then all plants grew five generations in control medium (without CuSO<sub>4</sub>). For analysis of performance and flavonoids the first daughter fronds of the fifth generation grown in control medium were grown in medium containing 20 µM CuSO<sub>4</sub> and the second daughter fronds were grown in control medium in 250 mL plastic beakers filled with 180 mL N-medium (“actual treatment”). After eight days the number of fronds was counted and the total fresh weight was determined. Samples of 50 mg were immediately frozen in liquid nitrogen in Eppendorf tubes and stored at -80°C until further analysis.

#### 4.2.4 Experimental Evolution of Copper Resistance

In order to test whether *S. polyrhiza* plants evolve copper resistance upon prolonged exposure to copper, replicated monoclonal *S. polyrhiza* populations (genotype 7498 USA) were cultivated in the presence and absence of 20 µM CuSO<sub>4</sub> (copper) over four months. Before the start of the experiment, plants were pre-cultivated in 18 L containers and equally distributed into 20 52 L transparent plastic ponds (79x58x17.5 cm) that were filled with 30 L N-medium. The ponds were covered with 4 mm transparent plexiglass with spaces of 5 mm for air circulation. One half of the ponds received 20 µM CuSO<sub>4</sub> (“background treatment”).

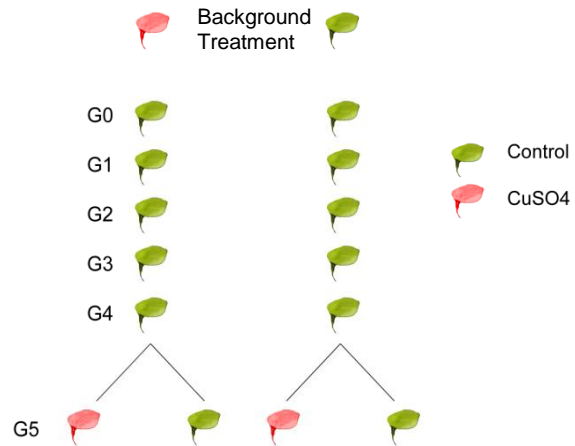
Every two weeks – when the control populations covered approximately the entire pond – plants covering about 5% of the total pond surface were randomly chosen and transferred into the cleaned and refilled ponds.

As algal growth inhibited reproduction of the duckweeds four weeks after start of the experiment, the ponds were connected to filters ( $6.7 \pm 0.2$  L water exchange rate) in week 19, which facilitated growth rates similar to starting conditions.

In the first four weeks of the experiment population size per growth pond reached approximately 27000 fronds in control medium, whereas in copper medium population size varied from 5000 to 16000 fronds with 0.12 to 0.25% brown fronds. When algae growth appeared in the media the maximum population size decreased to 10000 to 27000 fronds with 0.001% brown and white (dead) fronds in control medium. In copper media population size was still between 5000 and 17000 fronds with 0.2 to 0.6%

brown/yellow fronds. After installation of filters, to remove algae from water, population size of *Spirodela polyrhiza* returned back to approximately 27000 fronds and no dead fronds were observed anymore under control conditions. Under copper treatment population size was between 7000 and 11000 fronds, 0.3% of these fronds were brown. Therefore the assembly of a filter system was successful to restore the sterile starting conditions.

Four months after beginning of the experiment eight fronds (Generation 0) carrying small daughter fronds were taken from each pond and propagated for five generations in 30 mL control medium (no  $\text{CuSO}_4$ ) in 50 mL transparent plastic tubes ( $\varnothing$  2.8 cm, height 9.5 cm) covered with foam plugs. The propagation scheme is shown in Figure 4-2. Daughter fronds that had fully emerged were transferred to a new tube. For the “actual treatment” daughter fronds were selected as described in the following text. During the fifth generation five mother fronds of each pond were



**Figure 4-2: Propagation scheme for *Spirodela polyrhiza* plants to analyze plant fitness and flavonoid levels of copper evolved and non-evolved populations in the presence and absence of copper stress.** Independent of the background treatment all plants were cultivated in N-medium for generations G0 to G4.

selected and the first daughter fronds were transferred into 10 L control medium in 18 L plastic trays covered by a transparent plastic lid. The second daughter fronds of the same five mother fronds were transferred to the plastic trays filled with 10 L N-medium containing  $20 \mu\text{M}$   $\text{CuSO}_4$ . In order to account for algae that may have co-evolved copper resistance and thereby may affect duckweed growth, the trays were divided in the middle by a mesh. In every tray plants of a control experimental evolution pond were transferred to one half and those of a copper experimental evolution pond to the other half. Every second day deionized water was refilled to maintain the water level. After eight days the number of healthy (green) and sickly (brown) appearing fronds (criteria for brown fronds: 50% or more of frond surface brown) was counted and medium was changed to prevent nutrient limitation. After 16 days, the number of brown and green plants was counted again, harvested, weighed and 50 mg were frozen in liquid nitrogen in Eppendorf tubes and stored at  $-80^\circ\text{C}$  until further analysis.

#### 4.2.5 Flavonoid Extraction and Analysis

To measure flavonoid concentrations, the plant tissue was ground by vigorously shaking the Eppendorf tubes with two metal beads for 1 min in a paint shaker (ball mill) and extracted with 1 mL methanol per sample by thoroughly mixing for 10 s. All samples were centrifuged for 10 min at room temperature at 17,000 g. The supernatant was analyzed on an HPLC 1100 series equipment (Agilent Technologies, injection volume = 10  $\mu$ L) coupled to a photodiode array detector (G1315A DAD, Agilent Technologies). Analyte separation was accomplished with a Nucleodur Sphinx RP column (250  $\times$  4.6 mm, 5  $\mu$ m particle size, Macherey-Nagel). The mobile phase consisted of 0.2% formic acid (A) and acetonitrile (B) at a flow rate of 1 mL  $\cdot$  min<sup>-1</sup> using the gradient shown in Table 4-3, followed by column reconditioning.

To quantify the flavonoid concentration, the peak area of the four major flavonoids in *Spirodela polyrhiza* and anthocyanins was integrated at 330 nm and at 520 nm, respectively, and quantified based on external standards of luteolin-7-O-glucoside and kuromanin, respectively. For quantification of the four major flavonoids weight based response factors were used. For calculation of total flavonoid concentration only these four flavonoids were considered.

**Table 4-3: Concentration gradient of mobile phase on HPLC-UV/Vis;** solvent A = 0.2% formic acid, solvent B = acetonitrile

Time point [min]	Solvent A [%]	Solvent B [%]
8.0	79	21
17.0	45	55
17.1	0	100
18.0	0	100
18.1	90	10
22.0	90	10

#### 4.2.6 Statistical Analysis

For statistical analysis all data sets were evaluated in R (Version 3.3.1). Comparisons were performed in pairs, using Tukey's HSD test.

For analysis of heavy metal resistance experiment differences in the growth rate and biomass production, copper and control treated plants were analyzed with paired Student's *t*-test using the mean value of each genotype and treatment. Growth rate was calculated as  $(\ln(\text{nr fronds}_{\text{end}}) - \ln(\text{nr fronds}_{\text{start}}))/\text{nr growth days}$ . The correlation between growth rate, total number fronds, total fresh weight and flavonoid concentration in the presence and absence of  $\text{CuSO}_4$  were analyzed using linear models based on the mean value of each treatment and genotype. Differences in the total flavonoid concentration as well as the concentration of the four major flavonoids between copper and control-treated plants were analyzed with paired Student's *t*-test based on the mean value of each genotype and treatment. The correlation between control and copper-induced total flavonoid concentration was analyzed with a linear model. The correlations between relative fitness (based on number of fronds and biomass) and constitutive and copper induced flavonoid levels were analyzed with linear models. Relative fitness was calculated as the mean of the copper plants of genotype *i*/ mean of control plants of genotype *i*. As HPLC-based analysis revealed that genotype Sp76 produced different flavonoids than the other genotypes, this genotype was excluded from all analyses.

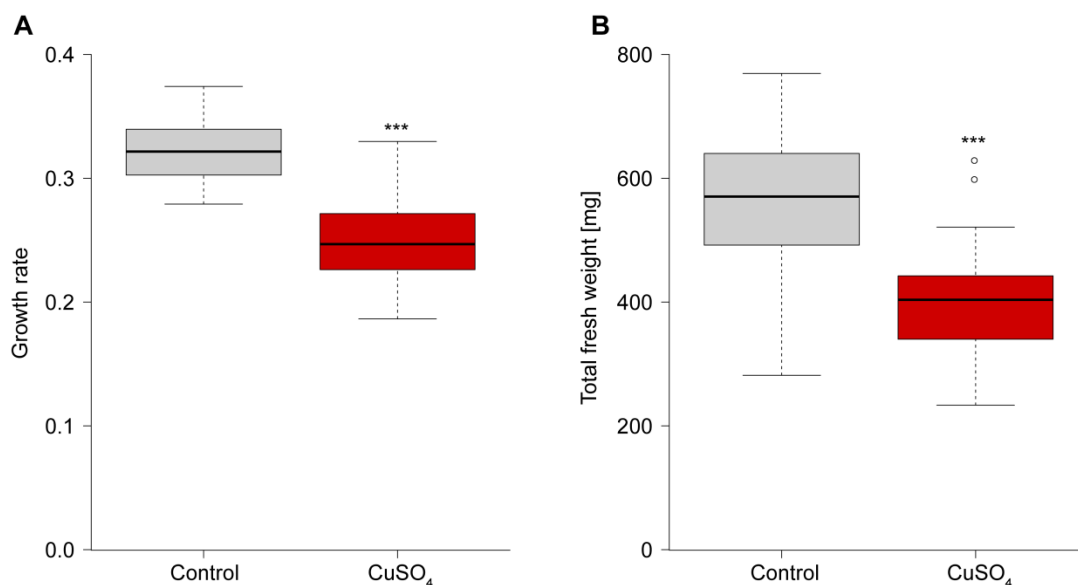
For experimental evolution of copper resistance differences in the number of fronds, fresh weight, concentration of the four major flavonoids (luteolin-8-C-, luteolin-7-O-, apigenin-8-C- and apigenin-7-O-glucoside) and the total flavonoid concentration between the different background and actual treatments were compared using two-way ANOVAs. As the interaction between background and actual treatment was not significant for total flavonoid, apigenin and luteolin glucoside concentrations, this term was removed from the final models of which values are reported. Growth rates over the entire experiment ("overall growth rate"), as well as between day 0 and day 8, and between day 8 and day 16 ("interval growth rates") were calculated as  $(\ln(\text{nr fronds}_t) - \ln(\text{nr fronds}_{t_{\text{foundingPopulation}}}))/dt$ , with *t* = day. Differences in the overall and interval growth rates were analyzed with two-way ANOVAs.

## 5 Results

### 5.1 Genotype Resistance

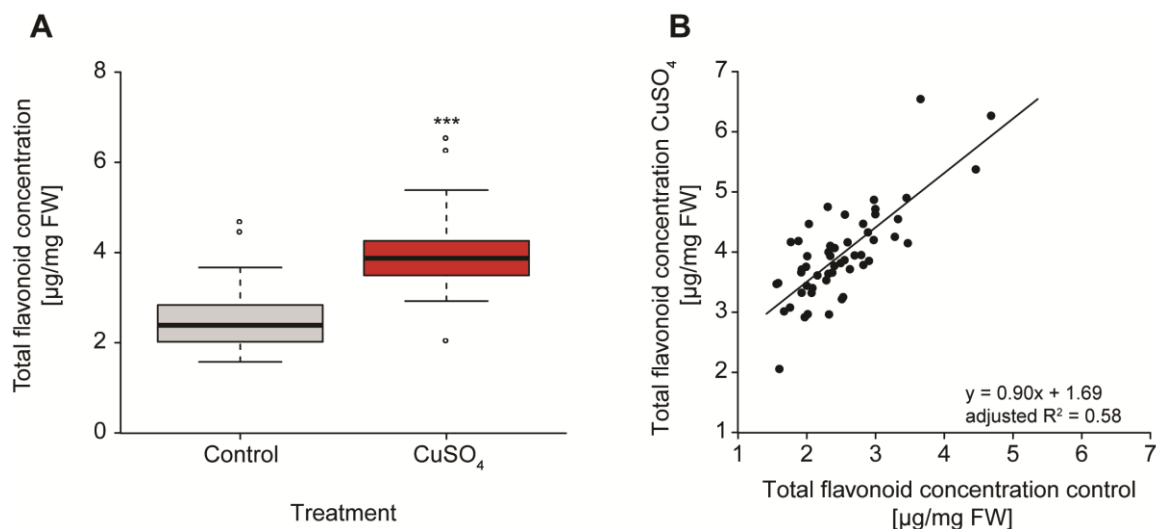
To determine whether variation in flavonoid levels of natural *Spirodela polyrhiza* genotypes correlates with plant resistance to copper stress, 53 genotypes were grown with and without 10  $\mu\text{M}$   $\text{CuSO}_4$  (“copper”) for seven days.

First, plant performance as measured via growth rate and total fresh weight was analyzed as second indicator. The growth rate in plants grown under copper treatment was slower, at a rate of 0.25  $\text{day}^{-1}$ , while controls grew at 0.32  $\text{day}^{-1}$ . This represents a 23% decrease (Figure 5-1A). Total fresh weight decreased from a mean of 565.8 mg under copper treatment in control plants to 398.5 mg (Figure 5-1B). Thus fresh weight decreased by 30% under copper stress.



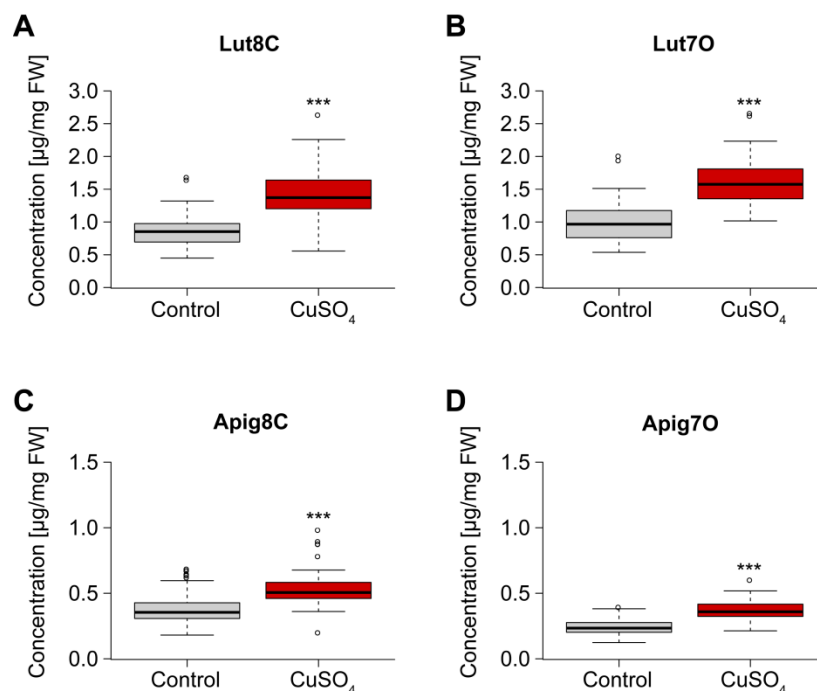
**Figure 5-1: Performance analysis of 53 *Spirodela polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$  after seven days of cultivation.** (A) Growth rate and (B) fresh weight of plants grown in copper medium decreased by 23% and 30%, respectively. The growth rate was calculated as  $(\ln(\text{nr fronds}_{\text{end}}) - \ln(\text{nr fronds}_{\text{start}})) / \text{nr growth days}$ . One data point represents the mean value of one clone and treatment ( $n = 3$ ). Data was analyzed with paired Student's  $t$ -test (\*\*\*) =  $P < 0.001$ .

In addition to plant performance the flavonoid concentrations were measured. Total flavonoid concentration increased from 2.51  $\mu\text{g}/\text{mg}$  fresh weight in control plants to 3.97  $\mu\text{g}/\text{mg}$  fresh weight in copper treated plants (Figure 5-2A). Thus total flavonoid concentration increases by approximately 60% under copper treatment. The total flavonoid concentration between control and copper-induced plants has a strong positive correlation (Figure 5-2B).



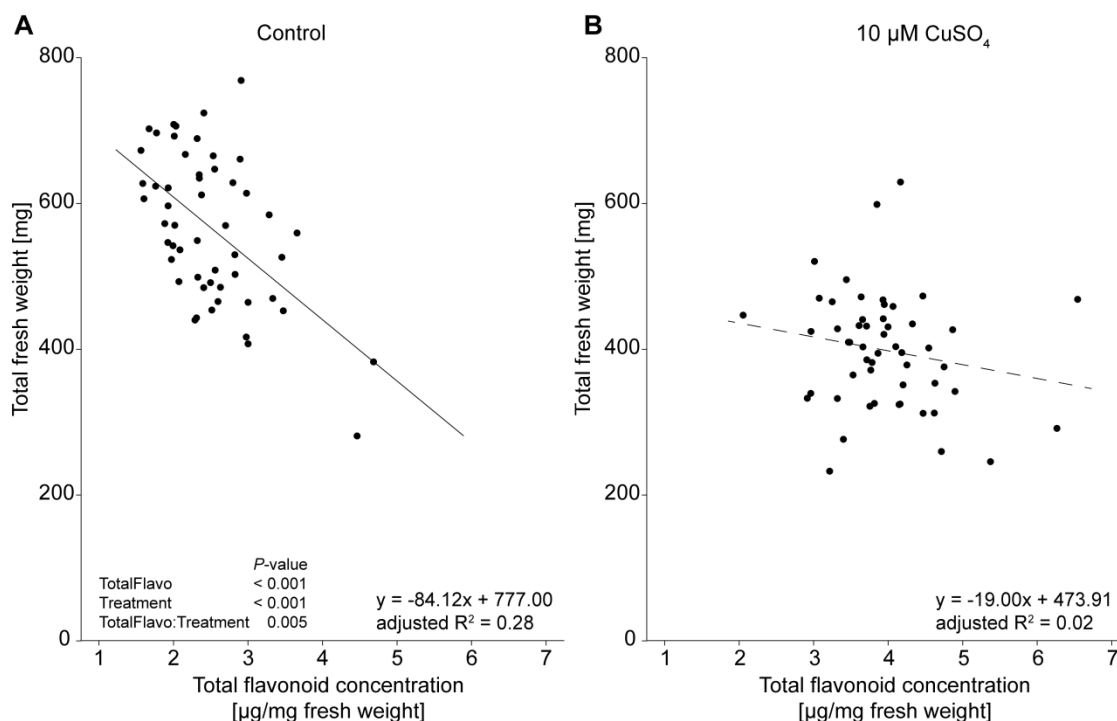
**Figure 5-2: Total flavonoid concentration across 53 *S. polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$  after seven days of cultivation.** One data point represents the mean of three biological replicates per clone and treatment. (A) Compared to controls total flavonoid concentration was increased in copper treated plants ( $P < 0.001$ ). Data was analyzed with Student's  $t$ -test. (B) Concentration of total flavonoids is positively correlated between control and copper (10  $\mu\text{M}$ ) treated plants across 53 *S. polyrhiza* genotypes ( $P < 0.001$ ). Statistics shown are from linear regression analysis. FW = fresh weight.

As shown in Figure 5-3 levels of luteolin-glucosides were higher than those of apigenin-glucosides in plants grown with and without  $\text{CuSO}_4$ . Under copper treatment the concentration of luteolin-8-C-glucoside (Figure 5-3A) increased from 0.89  $\mu\text{g}/\text{mg}$  fresh weight to 1.44  $\mu\text{g}/\text{mg}$  fresh weight. And the level of luteolin-7-O-glucoside (Figure 5-3B) increased from 1.00  $\mu\text{g}/\text{mg}$  fresh weight to 1.61  $\mu\text{g}/\text{mg}$  fresh weight under copper treatment. Whereas concentrations of apigenin-8-C-glucoside (Figure 5-3C) and apigenin-7-O-glucoside (Figure 5-3D) raised from 0.38  $\mu\text{g}/\text{mg}$  fresh weight and 0.24  $\mu\text{g}/\text{mg}$  fresh weight, respectively to 0.54  $\mu\text{g}/\text{mg}$  fresh weight and 0.37  $\mu\text{g}/\text{mg}$  fresh weight. Despite the different concentration levels the degree of induction was similar across all four major flavonoids (62-70% increase upon copper exposure). These four flavonoids correlated positively with each other (see supplement Figure 10-1).



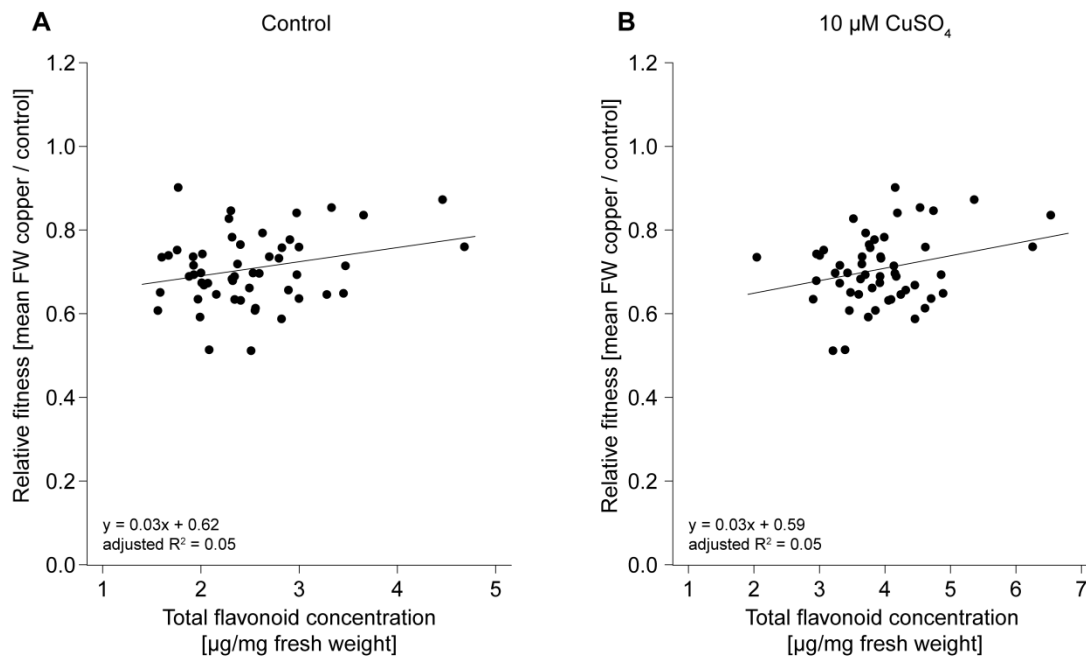
**Figure 5-3: Flavonoid concentration of the four major flavonoids across 53 *S. polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$  after seven days of cultivation.** The four major flavonoids ((A) luteolin-8-C-glucoside, (B) luteolin-7-O-glucoside, (C) apigenin-8-C-glucoside, (D) apigenin-7-O-glucoside) were increased in plants grown in  $\text{CuSO}_4$  medium. One data point represents the mean of three biological replicates per treatment and clone. Data was analyzed with paired Student's *t*-test (\*\*\*) =  $P < 0.001$ ). FW = fresh weight; Lut8C = luteolin-8-C-glucoside; Lut7O = luteolin-7-O-glucoside; Apig8C = apigenin-8-C-glucoside; Apig7O = apigenin-7-O-glucoside.

In order to test whether variation in the flavonoid levels correlates with copper resistance of *Spirodela polyrhiza* a linear regression analysis between the total flavonoid concentration and the total fresh weight of plants grown with and without copper treatment was performed. Total fresh weight of *S. polyrhiza* was found to be strongly negatively correlated with total flavonoid concentration in plants grown in control medium (Figure 5-4A). In contrast, no correlation between total flavonoid concentration and total fresh weight was observed in the plants grown in copper medium (Figure 5-4B). Statistics with ANOVA reveal a significant negative interaction between flavonoid concentration and treatment. A similar but less pronounced pattern was observed for the correlation of the number of fronds and total flavonoid concentration (see supplement Figure 10-2) and for the correlation of growth rate and total flavonoid concentration (see supplement Figure 10-3). Both, number of fronds as well as growth rate were negatively correlated with total flavonoid concentration in control medium grown plants. Whereas in copper treated plants the number of fronds as well as the growth rate did not correlate with total flavonoid concentration.



**Figure 5-4: Linear regression analysis between total flavonoid concentration and total fresh weight across 53 *S. polyrhiza* genotypes after seven days of cultivation in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$ .** (A) In control medium fresh weight was negatively correlated with flavonoid concentration ( $P < 0.001$ ; adjusted  $R^2 = 0.28$ ). (B) In copper medium linear regression analysis revealed no significant correlation between fresh weight and flavonoid concentration (adjusted  $R^2 = 0.02$ ). One data point represents the mean of three biological replicates per clone and treatment.  $P$ -values of a two-way ANOVA are shown.

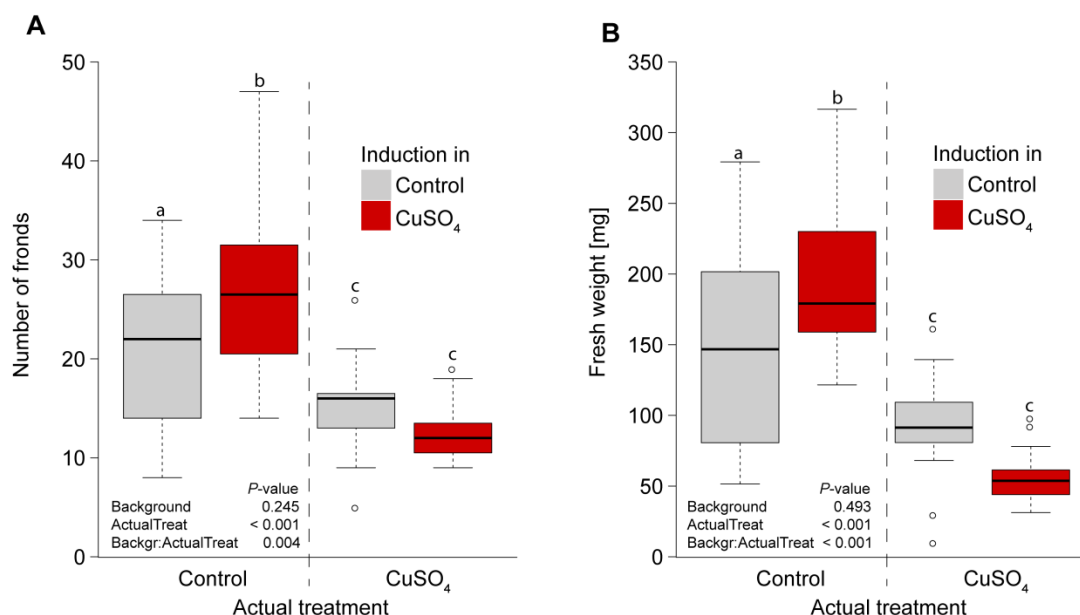
For closer examination of the role of the different flavonoid concentrations in plant resistance to copper stress (in *Spirodela polyrhiza*) relative plant fitness was determined for each genotype. Here the relative plant fitness was based on total biomass as well as the number of fronds. Relative plant fitness based on means of the total biomass production for each genotype showed a tendency towards a positive correlation with constitutive and copper induced flavonoid concentration (Figure 5-5). In contrast, relative plant fitness based on the number of fronds (mean number fronds copper/ control) was not correlated with constitutive or induced total flavonoid concentration (see supplement Figure 10-4).



**Figure 5-5: Linear regression analysis of biomass-based relative plant fitness across 53 *S. polyrhiza* genotypes with constitutive and induced total flavonoid concentration.** Relative plant fitness is expressed as mean fresh weight copper<sub>genotype i</sub> / control<sub>genotype i</sub>. Linear regression analysis revealed that relative plant fitness tended to be positively correlated with (A) constitutive ( $P = 0.06$ ) and (B) induced ( $P = 0.05$ ) total flavonoid concentration. One data point represents the mean of three biological replicates per clone and treatment. FW = fresh weight.

## 5.2 Transgenerational Inheritance

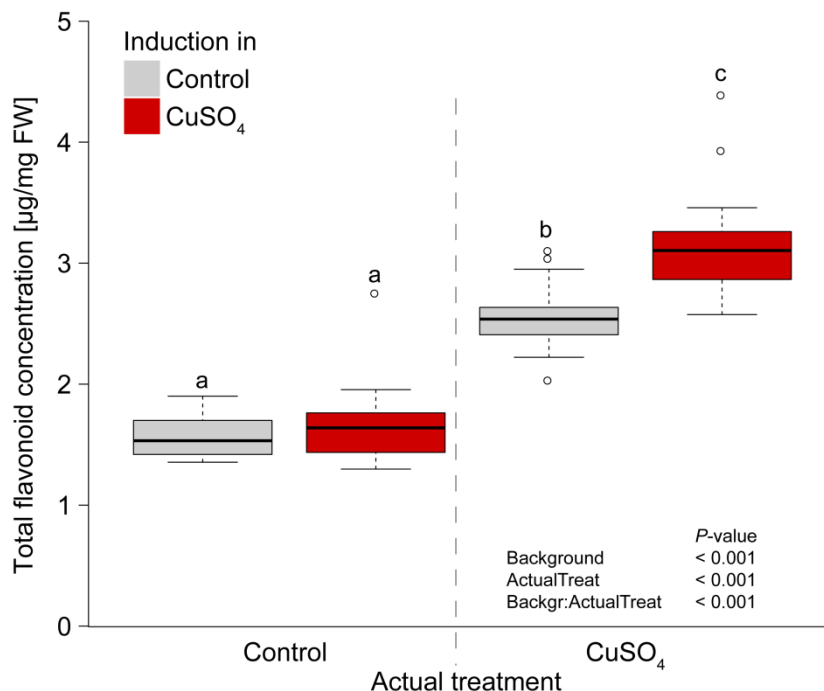
To investigate whether previous exposure to copper affects *Spirodela polyrhiza* offspring performance under the same stress condition, *S. polyrhiza* plants were grown for five generations in medium with or without 20  $\mu$ M CuSO<sub>4</sub> ("background treatment"). Afterwards plants were grown for five more generations in control medium to erase maternal effects. The daughter fronds of the fifth generation of both induction treatments were cultivated in control and 20  $\mu$ M CuSO<sub>4</sub> containing medium for eight days.



**Figure 5-6: Performance analysis of *S. polyrhiza* offspring cultivated in full N-medium with and without 20  $\mu$ M CuSO<sub>4</sub>.** (A) Number of fronds and (B) fresh weight of offspring grown in control medium was higher compared to plants grown in CuSO<sub>4</sub> medium. Offspring of copper-induced plants do not show an improved performance under the same stress conditions, but show increased performance under control conditions compared to non-induced plants. Different letters indicate a significant difference ( $P < 0.05$ ) according to a Tukey's honest significance test.  $P$ -values of a two-way ANOVA are shown.

Consistent with previously observed lower plant fitness caused by copper treatment, the number of fronds (Figure 5-6A) and plant fresh weight (Figure 5-6B) decreased during copper treatment. This was true for the control and copper-induced plants both when compared with plants grown in control medium as actual treatment. After eight days of growth control-induced plants showed a mean number of fronds of 21 under control-conditions, this number was reduced under copper-treatment to 15 in control-induced plants and to 12 in copper-induced plants (Figure 5-6A). Similar to this the mean fresh weight of 148.76 mg measured for control-induced plants in control treatment was reduced in control- and copper-induced plants under copper-treatment to 91.55 mg and 55.10 mg, respectively (Figure 5-6B). Thus, copper-induced plants performed slightly but

not significantly worse in the copper treatment than compared to control-induced plants. Interestingly, copper-induced plants performed significantly better in control medium but worse in copper medium when compared to the corresponding control-induced plants. With a mean number of 27 fronds and 195.40 mg fresh weight copper-induced plants showed the highest performance values within the four different treatment groups. As shown in supplement Figure 10-5, the fresh weight was positively correlated with number of fronds (linear model).



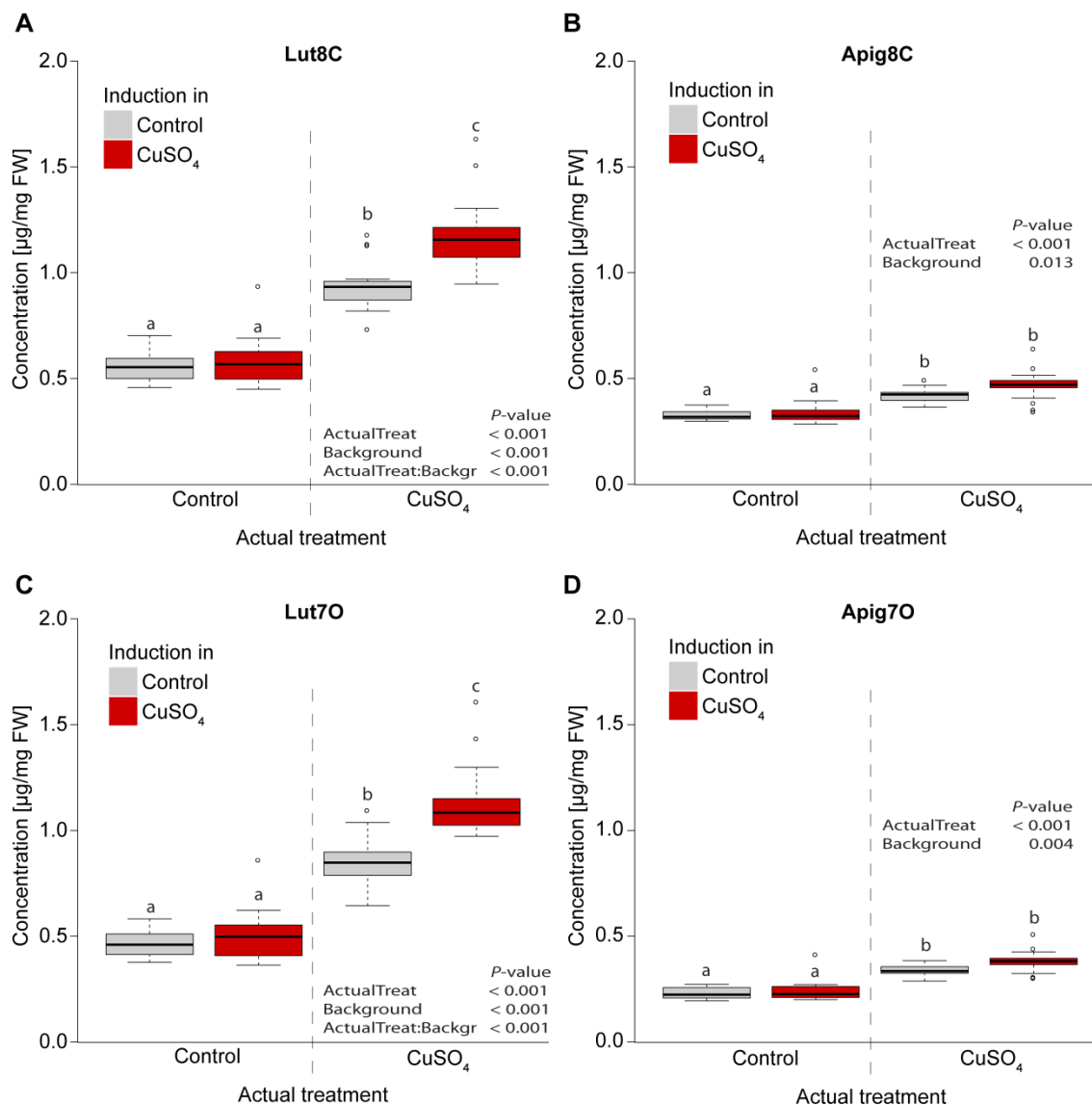
**Figure 5-7: Total flavonoid concentration of *S. polyrhiza* offspring cultivated in full N-Medium with and without 20 µM CuSO<sub>4</sub>.** The total flavonoid concentration was increased in offspring grown in CuSO<sub>4</sub> medium as actual treatment. The interaction between induction treatment and actual treatment has significant influence on the total flavonoid concentration. Different letters indicate a significant difference ( $P < 0.05$ ) according to a Tukey's honest significance test.  $P$ -values of a two-way ANOVA are shown. FW = fresh weight.

In addition to plant performance of copper-treated offspring under copper and control conditions the flavonoid concentrations were analyzed. Under copper stress but not under control conditions, plants from the copper background treatment had higher total flavonoid concentration than plants from the control background treatment (Figure 5-7). Total flavonoid concentration of control-induced plants increased from 1.57 µg/mg fresh weight under control conditions to 2.55 µg/mg fresh weight under copper treatment, which is an increase of 62%. However, copper-evolved plants showed an increase in total flavonoid concentration of 90%: from 1.66 µg/mg fresh weight with control treatment to 3.16 µg/mg fresh weight with copper treatment. Thus plants of both copper and control

background treatment showed an increased total flavonoid concentration when grown in copper containing medium as actual treatment.

All four major flavonoid concentrations increased under copper stress compared to control treatment (Figure 5-8). For example concentration of luteolin-8-C-glucoside (Figure 5-8A) increased in control-induced plants by 68% from 0.56  $\mu\text{g}/\text{mg}$  fresh weight to 0.94  $\mu\text{g}/\text{mg}$  fresh weight and in copper-induced-plants by 102% from 0.58  $\mu\text{g}/\text{mg}$  fresh weight to 1.17  $\mu\text{g}/\text{mg}$  fresh weight under copper treatment. Whereas the concentration of apigenin-8-C-glucoside (Figure 5-8B) increased in control-induced plants from 0.33  $\mu\text{g}/\text{mg}$  fresh weight to 0.42  $\mu\text{g}/\text{mg}$  fresh weight and in copper-induced plants from 0.34  $\mu\text{g}/\text{mg}$  fresh weight to 0.47  $\mu\text{g}/\text{mg}$  fresh weight, which is an increase of 27% and 38%, respectively.

Not only flavonoid but also anthocyanin concentration was increased in copper-induced plants compared to non-induced plants under the same stress conditions (see supplement Figure 10-6).



**Figure 5-8: Flavonoid concentrations of *S. polyrhiza* offspring cultivated in full N-medium with and without 20 µM CuSO<sub>4</sub>.** The four major flavonoids ((A) luteolin-8-C-glucoside, (B) apigenin-8-C-glucoside, (C) luteolin-7-O-glucoside, (D) apigenin-7-O-glucoside) were increased in plants grown in CuSO<sub>4</sub> medium (actual treatment). As the interaction between actual treatment and background treatment was not significant for apigenins (B and D), it was removed from the final model for these cases. Different letters indicate significant difference ( $P < 0.05$ ) according to a Tukey's honest significance test.  $P$ -values of two-way ANOVAs are shown. FW = fresh weight; Lut8C = luteolin-8-C-glucoside; Apig8C = apigenin-8-C-glucoside; Lut7O = luteolin-7-O-glucoside; Apig7O = apigenin-7-O-glucoside.

### 5.3 Experimental Evolution of Copper Resistance

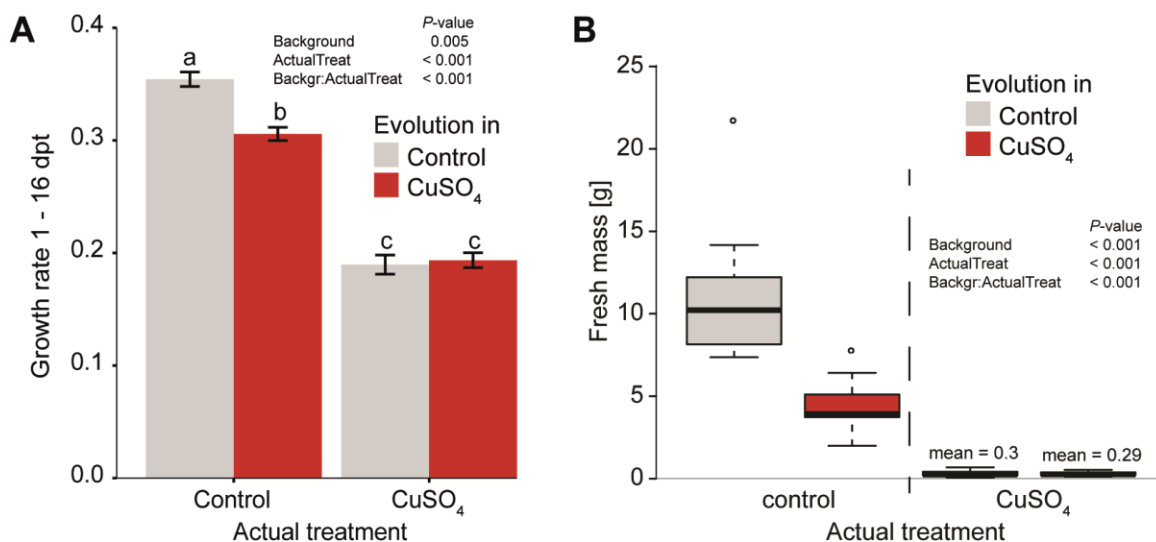
Since a short-time exposure to copper stress did not improve offspring performance under the same stress condition, the next step was to test whether long-term exposure results in an improved offspring performance.

To evolve copper resistance under laboratory conditions large populations of *S. polyrhiza* plants were grown for four months in N-medium with and without 20  $\mu\text{M}$   $\text{CuSO}_4$ . Afterwards the plants were grown for five generations in control medium after which the offspring were tested for differences in plant performance and flavonoid concentration after 16 days of cultivation.

#### 5.3.1 Growth Rates and Biomass Production

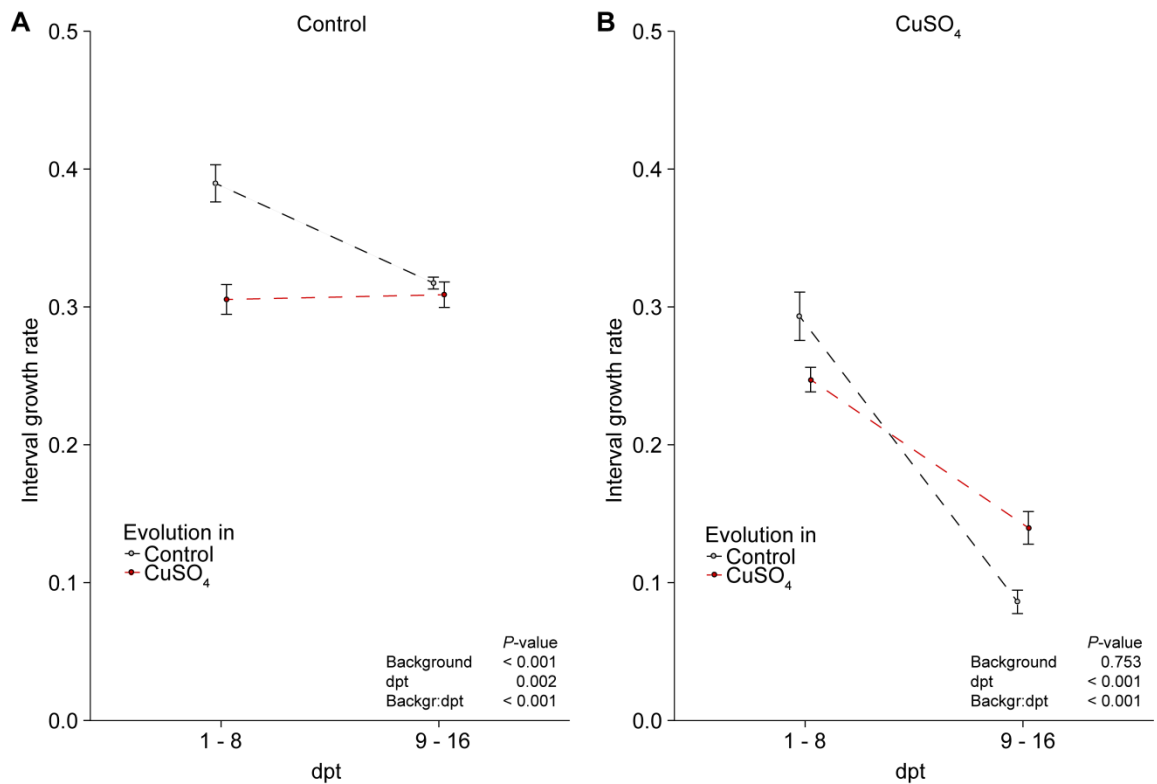
As shown in Figure 5-9, four months of copper exposure significantly affected the growth rate, based on total number of fronds as well as total biomass production. In the control medium, copper-evolved plants (mean = 4501 mg) produced less biomass than control-evolved plants (mean = 11424 mg) after 16 days of growth (Figure 5-9B). In contrast, in the copper medium no difference was observed in the biomass production of copper and control-evolved plants: plants of these treatments produced a mean fresh weight of 287 mg and 304 mg, respectively.

Overall the growth rate (Figure 5-9A) showed a similar pattern as the total biomass production. After 16 days of cultivation in the so-called actual treatment the growth rate was affected by copper. In the control medium, the copper-evolved plants (growth rate  $0.31 \text{ day}^{-1}$ ) grew significantly more slowly than the control evolved plants (growth rate  $0.35 \text{ day}^{-1}$ ). This is a decrease in the growth rate by 11%. In the copper medium, no difference in growth rates was observed ( $0.19 \text{ day}^{-1}$  for each treatment). Thus, the growth rate of control-evolved plants decreased by 46% under copper-stress, whereas the growth rate of copper-evolved plants decreased by only 39%.



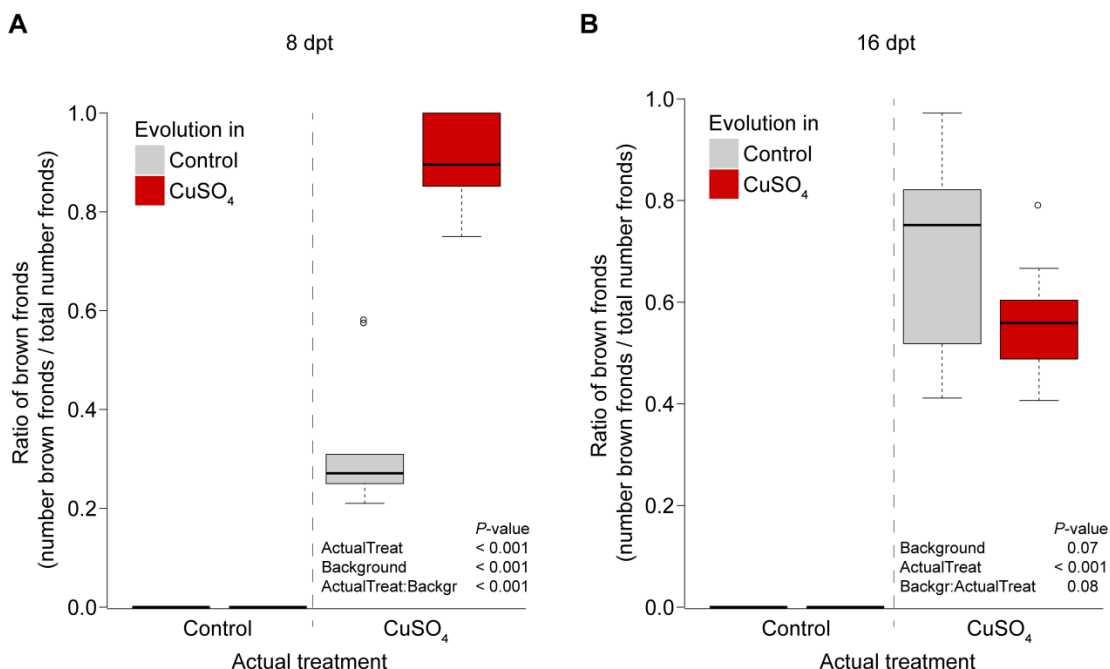
**Figure 5-9: Performance of copper (20  $\mu$ M) and control-evolved *S. polyrhiza* plants growing in copper (20  $\mu$ M) and control medium.** (A) Growth rate over the entire duration of the experiment. Values are means  $\pm$  standard error of five replicates each. Different letters indicate a significant difference ( $P < 0.05$ ) according to Tukey's honest significance test. (B) Biomass production of copper and control-evolved plants after 16 days of growth. The fresh-mass of copper-evolved plants was lower than control-evolved plants in control medium. In copper medium no difference in fresh was measured.  $P$ -values of two-way ANOVAs are shown. Dpt = days post transplantation.

Growth rates varied over time in a background-evolution dependent manner (Figure 5-10). In the first eight days of growth in the control medium (Figure 5-10A) the growth rate was lower in the copper-evolved plants ( $0.31 \text{ day}^{-1}$ ) compared to control-evolved plants ( $0.39 \text{ day}^{-1}$ ). Subsequently, the growth rate of control-evolved plants reached the level of the copper-evolved plants in control medium ( $0.32 \text{ day}^{-1}$  and  $0.31 \text{ day}^{-1}$ , respectively). In the copper medium (Figure 5-10B) the growth rate during the first eight days was lower for the copper-evolved plants than the control-evolved plants ( $0.25 \text{ day}^{-1}$  and  $0.29 \text{ day}^{-1}$ , respectively) - a pattern that was reversed in the subsequent eight days. Then growth rate of the control-evolved plants decreased by 69% to  $0.09 \text{ day}^{-1}$ , whereas growth rate of copper-evolved decreased only by 44% to  $0.14 \text{ day}^{-1}$ .



**Figure 5-10 Interval growth rates (1-8 dpt and 9-16 dpt) of copper and control-evolved plants in copper and control medium.** Interval growth rates were calculated as  $(\ln(nr\ fronds_t) - \ln(nr\ fronds_{t\_foundingPopulation}))/dt$ , with  $t = \text{day}$ . (A) Growth rate in control medium was higher in control-evolved plants during the first eight days and then reached a similar level as copper-evolved plants. (B) In copper medium the growth rate was higher in control-evolved plants during the first eight days of the experiment. In the subsequent eight days the growth rate decreased more in control-evolved plants than in copper-evolved plants. Therefore growth rate was higher in copper-evolved plants compared to control-evolved plants in copper medium during day 9 to 16 of the experiment. *P*-values are shown. Dpt = days post transplantation.

In addition to growth rate the ratio of brown fronds of copper evolved and non-evolved plants under copper stress was analyzed as an indicator for plant fitness. A similar time-dependent difference as observed for the growth rate was detected for the ratio of brown fronds (Figure 5-11). In the first eight days of growth in copper medium, almost all copper-evolved plants (91%) showed necrosis, whereas only about 30% of the control-evolved plants developed this symptom (Figure 5-11A). In contrast, after 16 days of growth in copper medium (Figure 5-11B), the copper-evolved plants tended to have a lower ratio of brown fronds (56%) than the control-evolved plants (69%). Plants grown in control medium did not show leaf browning during the entire 16 days of cultivation.

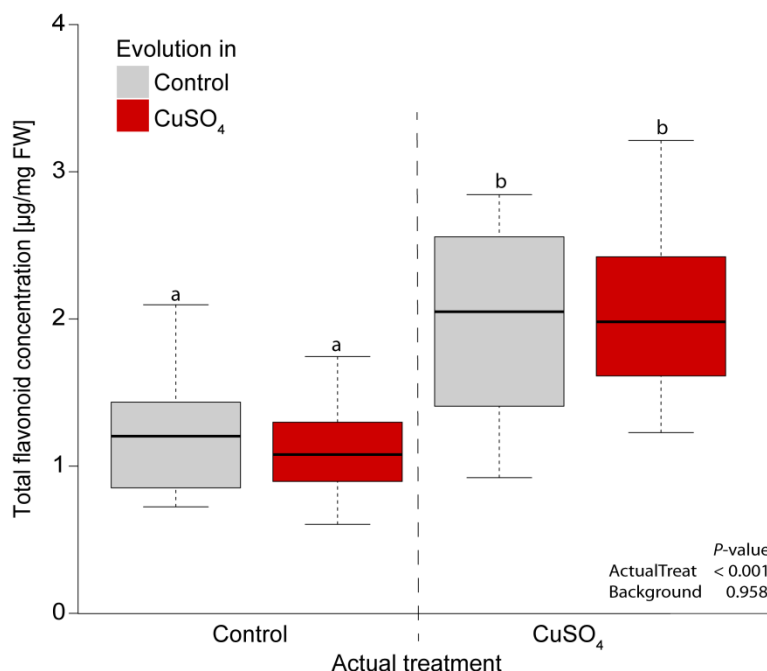


**Figure 5-11: Ratio of brown fronds of copper and control-evolved plants growing in copper and control medium.** (A) After 8 days of growth almost all copper-evolved plants showed necrosis in copper medium. In control medium no plants showed leaf browning (B) After 16 days of growth in copper medium the copper-evolved plants showed a lower ratio of brown fronds compared to control-evolved plants in copper medium. *P*-values of a two-way ANOVA are shown. Dpt = days post transplantation.

### 5.3.2 Flavonoid Concentrations

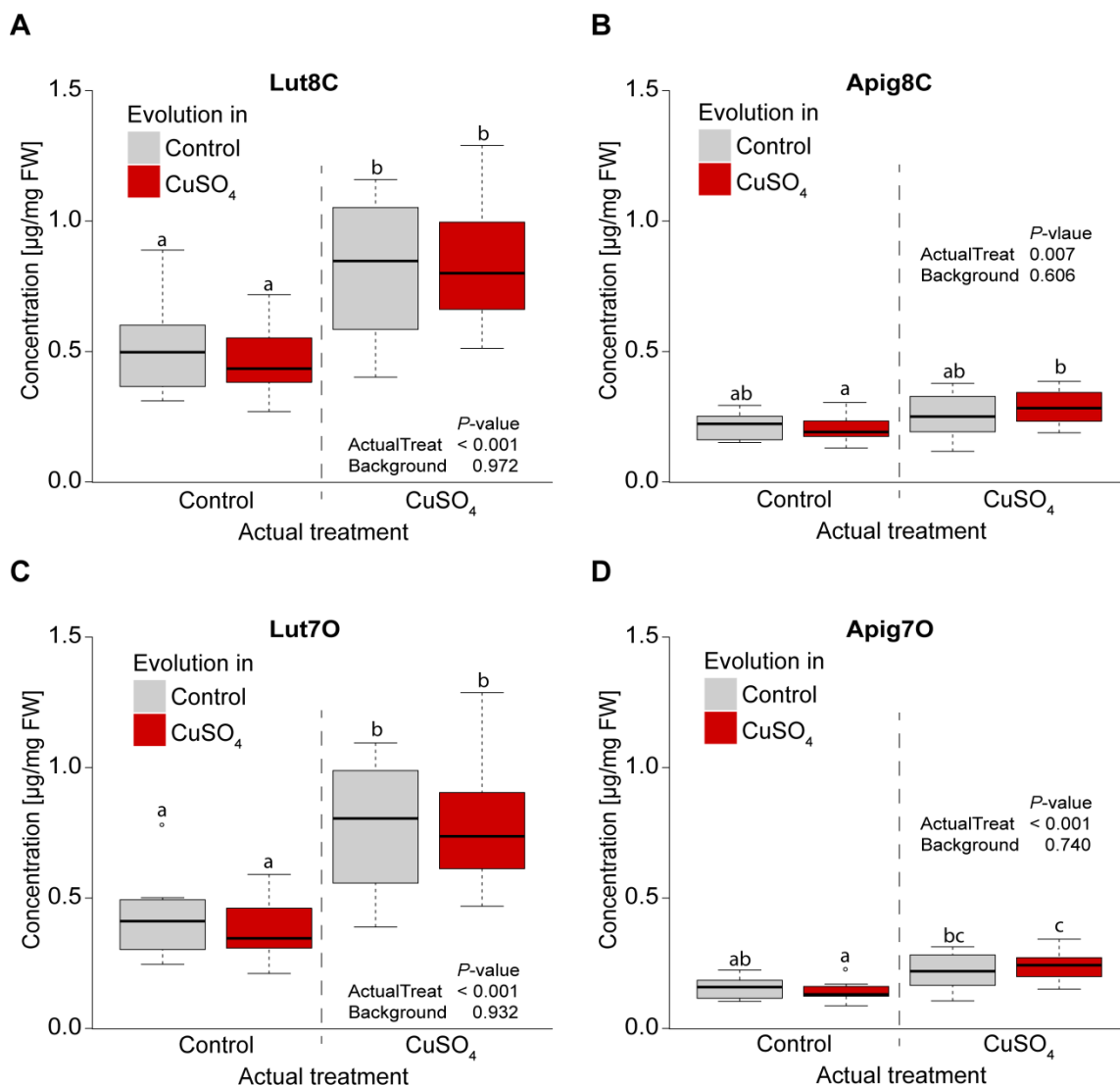
The focus during the investigation of the evolution of copper resistance in *Spirodela polyrhiza* was on the role of the flavonoids in the adaptation process.

The total flavonoid concentration increased during 16 days of growth in copper medium compared to control conditions as shown in Figure 5-12. Flavonoid concentration increased in control-evolved plants from 1.32 µg/mg fresh weight in control medium to 2.04 µg/mg fresh weight in copper medium. In copper-evolved plants flavonoid concentration increased from 1.19 µg/mg fresh weight under control conditions to 2.16 µg/mg fresh weight under copper stress. No significant difference between flavonoid levels of evolved and non-evolved plants could be observed under control-conditions and stress-conditions, respectively. Thus, evolution in copper medium did not affect the total flavonoid concentration compare to control-evolved plants.



**Figure 5-12: Total flavonoid concentration of copper and control-evolved *S. polyrhiza* plants after 16 days of growth in the presence and absence of 20 µM CuSO<sub>4</sub>.** Different letters indicate a significant difference ( $P < 0.05$ ) according to a Tukey's honest significance test.  $P$ -values of a two-way ANOVA are shown. FW = fresh weight.

A similar pattern as for total flavonoid concentration was observed for the individual four major flavonoids in *Spirodela polyrhiza* (Figure 5-13). Similar to previous analysis of plants that were short-time exposed to copper stress the luteolin-glucosides showed 2-fold higher levels than the apigenin-glucosides. The concentration of all four flavonoids increased in evolved and non-evolved plants under copper treatment. For example luteolin-8-C-glucoside concentration (Figure 5-13A) increased in non-evolved plants from 0.52 to 0.81 µg/mg fresh weight, but also in copper-evolved plants from 0.47 to 0.84 µg/mg fresh weight. This is an increase by 56% and 79%, respectively. The concentration of apigenin-8-C-glucoside (Figure 5-13B) increased in non-evolved plants from 0.21 to 0.25 µg/mg fresh weight and in copper-evolved plants from 0.20 to 0.29 µg/mg fresh weight. Thus, the apigenin-8-C-glucoside levels increased by only 19% and 45%, respectively.



**Figure 5-13: Concentration of the four major flavonoids of copper and control-evolved *S. polyrhiza* plants after 16 days of growth in the presence and absence of 20  $\mu\text{M}$  CuSO<sub>4</sub>.** The four major flavonoids ((A) luteolin-8-C-glucoside, (B) apigenin-8-C-glucoside, (C) luteolin-7-O-glucoside, (D) apigenin-7-O-glucoside) were increased in plants grown in CuSO<sub>4</sub> medium (actual treatment). Different letters indicate a significant difference ( $P < 0.05$ ) according to Tukey's honest significance test.  $P$ -values of two-way ANOVAs are shown. FW = fresh weight; Lut8C = luteolin-8-C-glucoside; Apig8C = apigenin-8-C-glucoside; Lut7O = luteolin-7-O-glucoside; Apig7O = apigenin-7-O-glucoside.

## 6 Discussion

This study provides evidence that flavonoids benefit plant fitness under copper stress. Furthermore, it was shown that exposure to copper for a few generations affects plant fitness and flavonoid levels of offspring under the same stress condition. Finally and for the first time, evidence was provided that long-term exposure of genetically homogenous *S. polyrhiza* populations to copper results in the evolution of copper resistance. Together, these data provide a framework to study past and on-going evolutionary processes in asexual plants.

### 6.1 Flavonoids benefit Duckweed Performance under Copper Stress

This study provides evidence that flavonoids benefits plant fitness under copper stress. Relative plant fitness expressed as fitness of copper-challenged plants relative to non-challenged controls was positively correlated with both constitutive and copper induced flavonoid concentrations across 53 *S. polyrhiza* genotypes. Interestingly, this pattern was mainly caused by a strong negative correlation between plant fresh weight and total flavonoid concentration under control conditions, a pattern that was erased under copper stress. Negative correlations between fitness and metabolites are usually taken to infer a cost of these compounds. Although costs are often assumed for secondary metabolites, they have remained difficult to demonstrate with a few examples of negative correlations between growth and metabolite production [34-37], and many examples without any correlation [38, 39]. Costs may include direct costs, e.g. due to the production of the compounds, and also indirect costs, for example they may negatively affect the interaction of an organism with beneficial symbionts [40]. Although indirect costs of flavonoid production cannot be ruled out, this study supports the hypothesis that flavonoids involve direct costs, as the experiments were performed largely in the absence of other organisms.

It has been previously reported that excess copper increased flavonoid concentrations in plants, for example in safflower (*Carthamus tinctorius*) [11]. In addition copper stress up-regulated the enzymes involved in the flavonoid biosynthesis such as PAL and the chalcone synthase in grapevine [12]. Furthermore, *A. thaliana* seedlings deficient in flavonoids show impaired growth under heavy metal stress, which could partially be restored when the flavonoids quercetin and naringenin were supplemented [10]. These

studies indicate that flavonoids play a role in heavy metal stress responses and could benefit plant performance under copper stress.

The mechanism behind the reduced toxicity of copper by flavonoids is not fully understood, but may be due to their function as potent ROS scavengers. Copper is known to induce ROS stress in plants [14]. Furthermore it is also possible that  $\text{Cu}^{2+}$  ions are directly bound by the flavonoids [5, 13]. Thus, it has been suggested that *ortho*-dihydroxylated B-ring flavonoids like luteolin are more important than their monohydroxylated counterparts as antioxidants due to their potential to form complexes with heavy metals [41]. In accordance to this hypothesis, flavonoids with high antioxidant potential are more strongly induced by oxidative stresses such as UV light compared to flavonoids with low antioxidant potential [42, 43]. In this study, contrasting results about the induction levels of luteolin glucosides (potentially high anti-oxidative capacity) and apigenin glucosides (potentially low anti-oxidative capacity) were obtained. Whilst luteolin and apigenin glycosides were similarly induced across 53 *S. polyrhiza* genotypes under copper stress; luteolin glucosides were more strongly induced than apigenin glucosides under copper stress in offspring of *S. polyrhiza* whose parents were exposed to copper previously. Targeted manipulation of luteolin and apigenin glucosides through either genetic or chemical engineering may provide further insights into the role of these compounds in copper resistance.

## **6.2 Copper Exposure induced Transgenerational Phenotypic and Fitness Effects in Copper-challenged Offspring**

During the last two decades, it has become increasingly clear that an organism's phenotype and fitness is not only determined by its genotype, environment and their interaction, but also by its epigenetic status [18, 44-47]. For example in apomictic dandelion (*Taraxacum officinale*) the same stresses that triggered heritable DNA methylation changes also caused transgenerational effects on offspring phenotypes [23, 48]. Environmental stresses induced DNA methylation changes at a genome-wide scale and many of these changes were transmitted (74-92%) to unexposed offspring [23]. As the environment may affect the epigenetic makeup of an organism, it has been suggested that environment-triggered epigenetic variation may lead to rapid adaptation if the induced traits are beneficial and stably transmitted over multiple generations [49].

In this study transgenerationally transmitted phenotypic and fitness effects were tested by cultivating *S. polyrhiza* plants for five generations with and without copper stress.

Subsequently, plants were grown five more generations under control conditions to reduce copper-induced physiological effects, after which offspring performance and flavonoid levels were analyzed under control as well as under copper stress conditions. Offspring of copper stressed plants showed improved performance in control medium but decreased fitness under the same stress conditions, contrary to the expectations of adaptive transgenerational effects. Two mechanisms may account for the seemingly maladaptive response. In the first, the observed pattern may have an epigenetic basis which results in fitness effects contrary to the expectations. In the second, maternal effects e.g. nutritional provision or hormonal signaling from mother to offspring may account for the observed pattern. Maternal effects may be particularly important in *S. polyrhiza*, as an emerging frond can contain already three daughter generations and the fronds stay linked through a thin connection that may allow nutrient and information exchange, called a stipe. Although five generations in control conditions were propagated prior to testing for transgenerationally inherited effects, and the connecting stipe between the fronds was removed, the role of such maternal effects cannot be ruled out completely. Therefore, this experiment is to be repeated with more generations in control medium before transferring the plants into the medium of the actual treatment. Furthermore, an experiment using demethylation agents such as zebularine [50, 51] may further provide insights into whether the observed fitness effects may have an epigenetic basis.

Interestingly, although the experiment did not indicate any adaptive transgenerational effects based on plant fitness, it was observed that offspring of copper stressed plants induced certain flavonoids to a greater extent than offspring from non-stressed plants under similar stress conditions. The increased induction was mainly due to luteolin-7-O-glucoside and luteolin-8-C-glucoside, in line with the hypothesis that such *ortho*-dihydroxylated flavonoids are more important for anti-oxidative stress reduction than their monohydroxylated counterparts [13, 52]. The difference in the structure between apigenin and luteolin merely consists in one additional hydroxyl group of the B-ring of luteolin [33], which results in enhanced metal chelating and antioxidative capacity (Figure 3-3) [13, 52]. Thus, an increase in luteolin-glucosides in plants cultivated in copper medium is a possible adaption to copper stress.

### **6.3 Evolution on Copper affects Plant Fitness under Copper in a Time-dependent Manner**

Experimental evolution studies have provided many insights into the progress of adaptations in short-lived organisms such as bacteria, fungi and certain animals. In contrast, experimental evolution studies using genetically homogenous plant populations have remained scarce due to the long generation time and large size of these organisms. Here large monoclonal populations of *S. polyrhiza* were cultivated under copper stress to test whether resistance to an environmental stress can evolve over approximately 40 generations in this small and rapidly reproducing plant. The results indicate that evolution of four months on copper affected plant fitness both in a copper- and time-dependent manner. Over the first eight days of growth, copper evolved plants had lower growth rates (between days 1-8) than control-evolved plants under both copper and control conditions. In contrast, during the subsequent eight days, the copper-evolved plants showed higher growth rates (between days 8-16) than control evolved plants under copper stress, and similar growth rates under control conditions. Although the final plant fitness did not differ between copper and control evolved plants under copper stress, the different interval growth rates of the copper and control-evolved plants indicate that evolutionary processes may have taken place that affect plant fitness. The performance data also contrasted the transgenerational experiment, in which short-term exposure to copper benefited plant fitness under control conditions during the first eight days of offspring growth, supporting the hypothesis that long-term exposure of copper affected the growth of these plants. However, these data also indicate that plant fitness needs to be investigated over longer time periods. Therefore, this experiment will be repeated over a longer period, and growth will be documented every second day. In a concerted effort we will simultaneously analyze for copper-induced transgenerational effects using plants from control background, which will facilitate the disentanglement of transgenerationally induced responses from evolved responses. Further experiments involving transcriptomics, whole-genome sequence analysis and whole genome bisulfite sequencing may provide further insights into the underlying mechanisms of the observed fitness differences of copper and control-evolved populations.

## 7 Outlook

While the diverse physiological and ecological functions of secondary metabolites are relatively well understood, the evolution of these metabolites remains largely unknown. Several key questions in the evolution of the metabolites remain to be investigated: (1) do environmental stresses act synergistically or antagonistically on the evolution of the metabolites? (2) what are the genetic loci under selection? (3) is the epigenome also under selection?

To date, a major bottleneck for these studies is the lack of an experimental system that allows to link genotype to phenotype to plant fitness in various environments. Taking advantage of the unique features of *Spirodela polyrhiza* may contribute to a deeper understanding of these outstanding questions and may reveal novel mechanisms of adaptations in clonal plants.

## 8 Acknowledgments

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## 10 Supplement

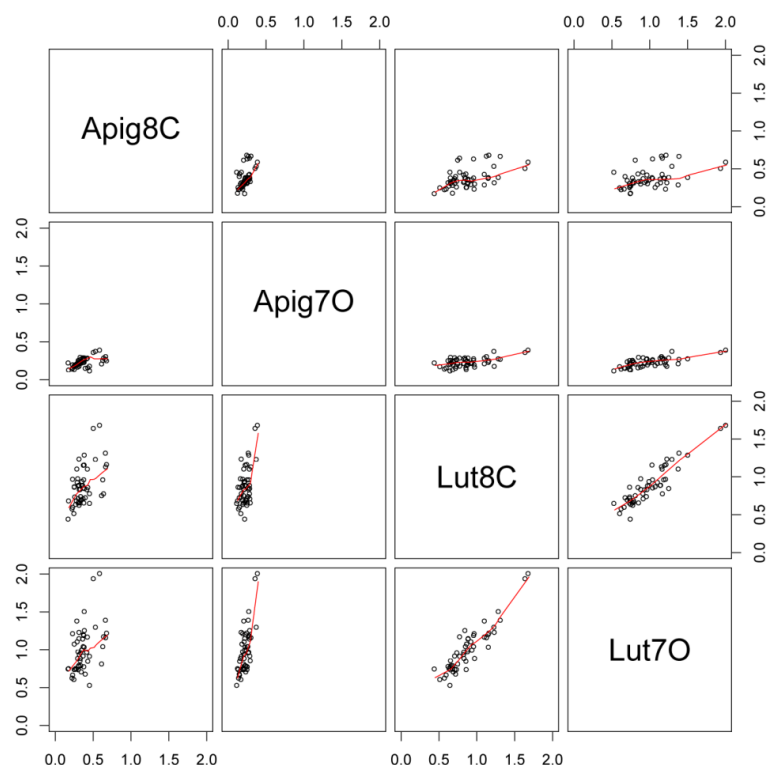
**Table 10-1: Utilized water pots and corresponding lids for plant cultivation**

Equipment	Name/Specifications	Company
Plastic beaker	Ø 9.6 cm, height 5 cm (250 mL), transparent PP	Plastikbecher.de GmbH (Giengen an der Brenz, Germany)
Plastic beaker lid	Ø 101.0 mm, transparent PP, perforated	Plastikbecher.de GmbH (Giengen an der Brenz, Germany)
Plastic tray	55x30x11 cm (18 L), dark green PP	Pöppelmann (Lohne, Germany)
Plastic tray lid	59x39-28x14 cm, transparent PET	Pöppelmann (Lohne, Germany)
Plastic box	4.9x3.4x3.8 cm per well, 4x10 wells, transparent	Voelkner (Nürnberg, Germany)
Plastic box lid	Transparent, 1 mm spacer	Voelkner (Nürnberg, Germany)
Pond	79x58x17.5 cm (52 L), transparent plastic	Regalux (BAUHAUS, Mannheim, Germany)
Pond lid	85x62.5 cm, transparent plexiglass, 5 mm spacer	
Tubes	Ø 2.8 cm, height 9.5 cm (50 mL), transparent	Kisker BIOTECH GmbH & Co KG (Steinfurt, Germany)

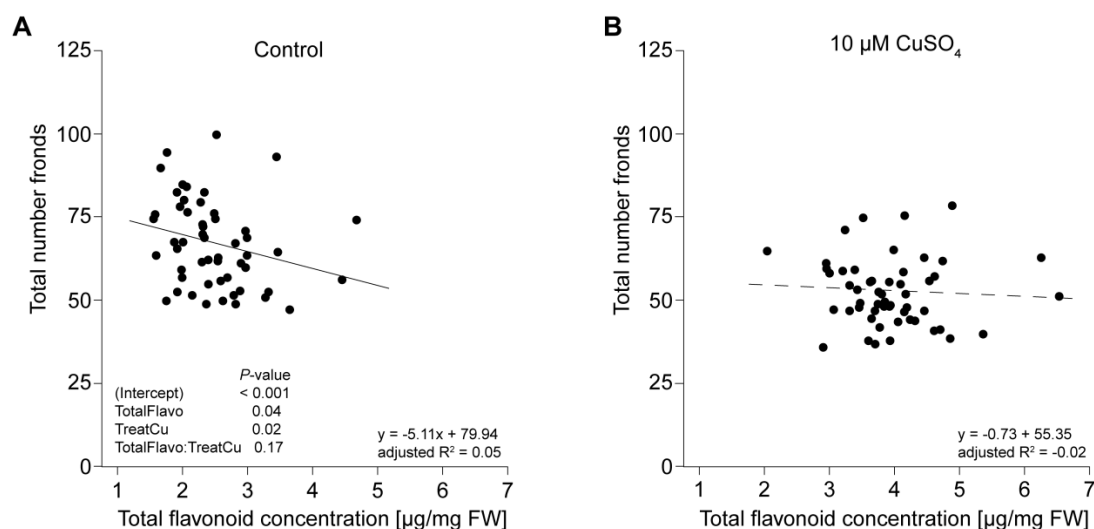
**Table 10-2: *S. polyrhiza* genotypes applied to examine whether flavonoid production correlates to plant fitness under copper stress.** Genotype Sp76 (grey) was removed from all analysis.

Genotype	AccNr	Continent	Country	Province/City
1	40	Asia	China	Sichuan, Xinjin
2	6613	North America	USA	California, Fresno Co., Centerville
3	7379	Asia	India	Tamil Nadu, Pondicherry
4	7498	North America	USA	North Carolina, Durham Co., Durham
5	7551	Australia	Northern Territory	Roper R., Mataranka
6	7652	Central America	Mexico	Tabasco, Villahermosa
7	7674	Asia	Nepal	Kathmandu
8	8683	Africa	Kenya	Meru Nat. Park
9	8756	Africa	Ethiopia	Wollo, Lake Maybahr
10	8790	North America	Canada	British Columbia, Vanderhoof
11	9242	South America	Ecuador	Guayas, Yaguachi Nuevo
12	9503	Asia	India	Rajasthan, Bharatpur, Bird Sabctuary, 2007
13	9507	Asia	Russia	Sibirien, Irkutsk
14	9509	Europe	Germany	Lotschen, Stadtroda 2002
15	9510	Africa	Mozambique	
16	9636	Asia	China	Kunming
17	9907	Asia	Bangladesh	Dhaka, Tangail
18	9511	Asia	Russia	Moscow
20	9625	Europe	Albania	Keneta e Zeze (Black marsh), Roskoveci, near Fieri
21	9500	Europe	Germany	Jena, Porstendorf 1967
22	9628	Europe	Albania	Berdice, Skodra district
23	9618	Europe	Italy	Trasimento lake, Perugia (43o 97'50" N)
25	9351	Asia	Vietnam	Hanoi, Tuliem
28	8118	North America	USA	Texas, Davis Co., Tohyavale
30	8442	Asia	India	Bihar, Patna
31	9514	Europe	Austria	Wien
32	9505	South America	Cuba	Havanna-Cuba, via Cunnersdorf GER 1977
33	7960	North America	USA	Tennessee, Obion Co., Reelfoot L.
34	9502	Europe	Ireland	Athlone
36	92	Asia	China	Jiangsu, Wuxi
38	Sp1	Asia	China	Hainan

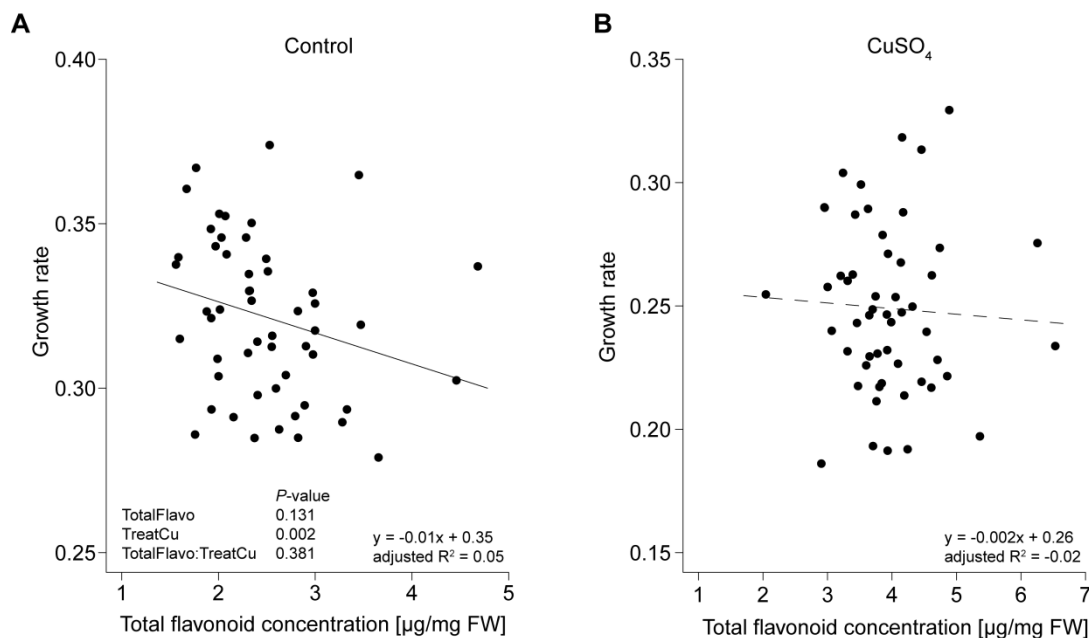
Genotype	AccNr	Continent	Country	Province/City
39	9650	Asia	India	Garhmukteshwar, Nakkakuan, Uttar Pradesh
40	9295	Asia	India	West Bengal, Calcutta, Bidhannagar
41	9609	Europe	Poland	Bialowieza lake, Bilalowieza, neare Belarus border
45	9512	Asia	Russia	Russia, Shelekhov, flood territory of Olkha river, ephemeral pond
48	9305	Asia	India	Andhra Pradesh, Hyderabad, Husainsagar
50	90	Asia	China	Sichuan, Jianyang
52	8403	Europe	France	Gironde, Bordeaux, Marais de Blanquefort
53	9290	Asia	India	Delhi, Shaheen Bagh
54	8787	Asia	Nepal	Kathmandu
56	9256	Europe	Finland	Uusimaa, Pukila
61	225	Asia	China	Yunnan, Kunming
62	9513	Europe	Czechia	Trebon
63	13	Asia	Vietnam	Hanoi
64	7657	Central America	Mexico	Veracruz, Coatzacoalcos
65	9413	Europe	Italy	Veneto, Po Delta
69	6731	North America	USA	Oregon, Douglas Co., Tahkenitch L.
73	8409	North America	USA	Arkansas, Crittenden Co., Wapanocca
75	9668	Asia	China	Sichuan, Chengdu, Shengxian Lake
76	7003	North America	USA	Louisiana, Terrebonne Par., Morgan City
77	9508	Europe	Poland	Krakau
78	9607	Europe	Switzerland	Zurich, Area of the Rämibühl school
79	Huenfeld	Europe	Germany	Huenfeld, Rhoen
81	5501	Europe	Hungary	Lake Kis-Balaton



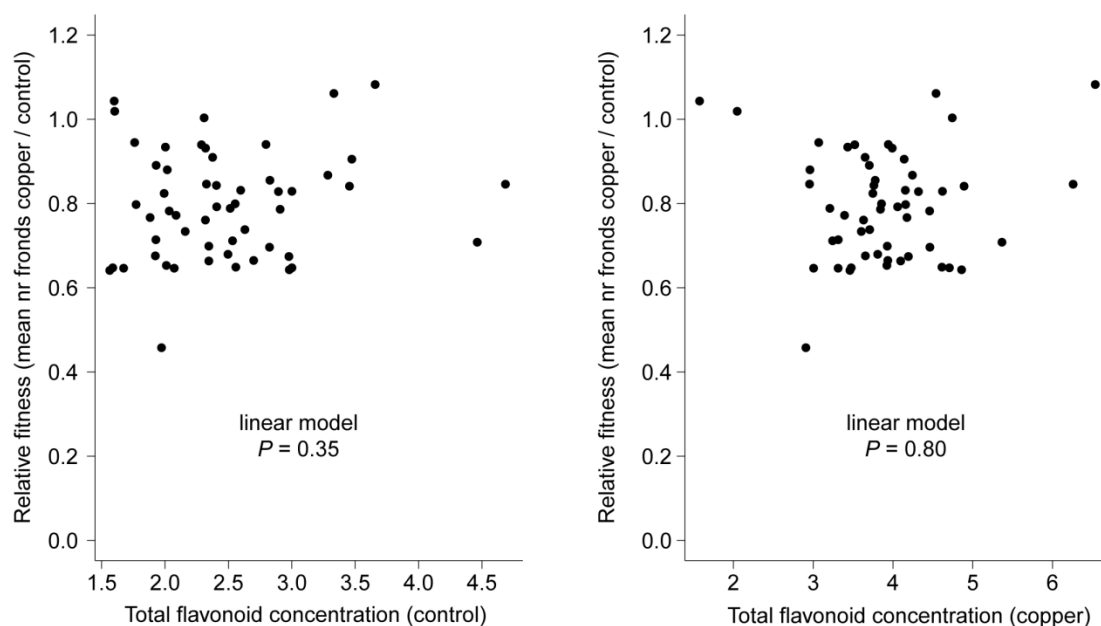
**Figure 10-1: Correlation of the four major flavonoids across 53 *S. polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$  after 7 days of growth.** Apig8C = apigenin-8-C-glucoside; Apig7O = apigenin-7-O-glucoside; Lut8C = luteolin-8-C-glucoside; Lut7O = luteolin-7-O-glucoside.



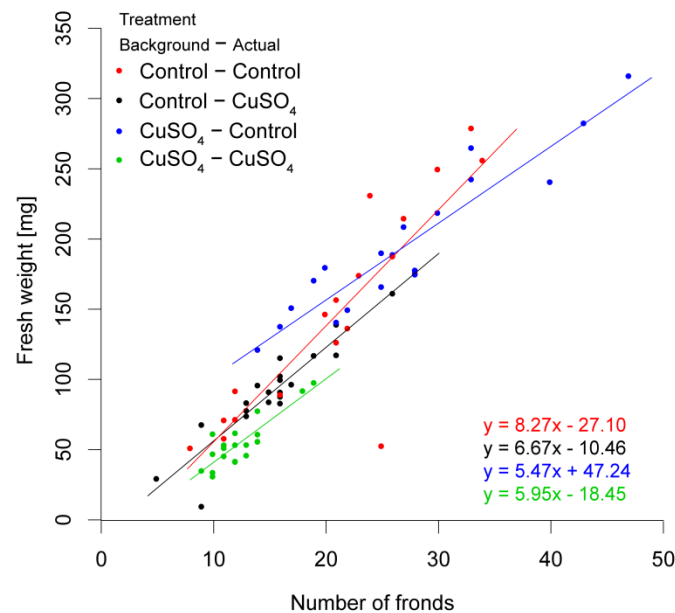
**Figure 10-2: Correlation between total flavonoid concentration and total number of fronds across 53 *S. polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$   $\text{CuSO}_4$  after 7 days of growth.** (A) In control medium total number of fronds was negatively correlated with total flavonoid concentration ( $P = 0.06$ ; adjusted  $R^2 = 0.05$ ). (B) In copper medium linear regression analysis revealed no significant correlation between number of fronds and total flavonoid concentration (adjusted  $R^2 = -0.02$ ). One data point represents the mean of three biological replicates per clone and treatment.  $P$ -values of a two-way ANOVA are shown. FW = fresh weight.



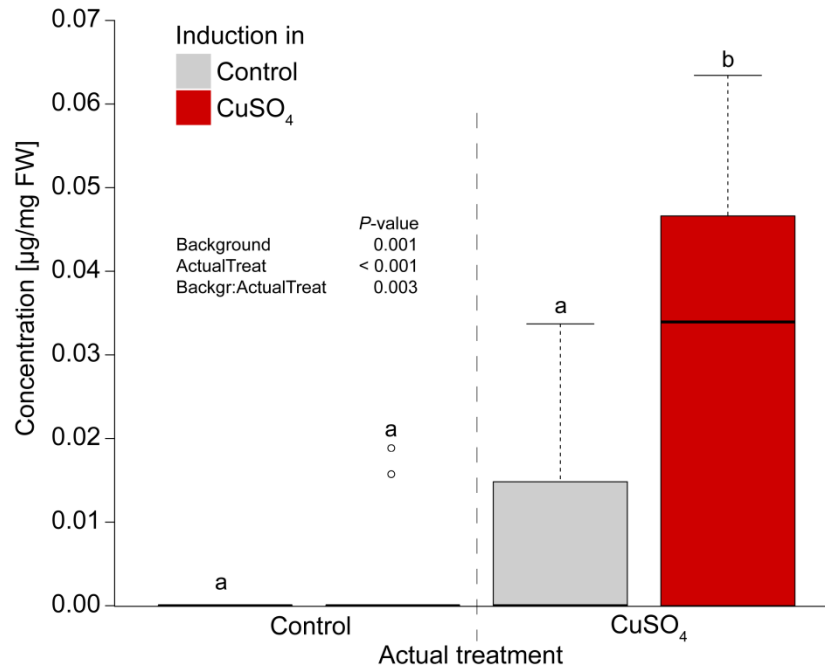
**Figure 10-3: Correlation between total flavonoid concentration and growth rate across 53 *S. polyrhiza* genotypes in the presence and absence of 10  $\mu\text{M}$  CuSO<sub>4</sub> after 7 days of growth.** Growth rate was calculated as  $(\ln(\text{nr fronds}_{\text{end}}) - \ln(\text{nr fronds}_{\text{start}}))/\text{nr growth days}$ . (A) In control medium growth rate was negatively correlated with total flavonoid concentration ( $P = 0.06$ ; adjusted  $R^2 = 0.05$ ). (B) In copper medium linear regression analysis revealed no significant correlation between growth rate and total flavonoid concentration ( $P = 0.71$ ; adjusted  $R^2 = -0.02$ ). One data point represents the mean of three biological replicates per clone and treatment.  $P$ -values of a two-way ANOVA are shown. FW = fresh weight.



**Figure 10-4: Correlation of number of fronds-based relative plant fitness (mean nr fronds copper<sub>genotype i</sub> / control<sub>genotype i</sub>) across 53 *S. polyrhiza* genotypes with (A) constitutive and (B) induced total flavonoid concentration.** One data point represents the mean of three biological replicates per clone and treatment. FW = fresh weight; nr = number.



**Figure 10-5: Linear regression analysis between fresh weight and number of fronds of *S. polyrhiza* offspring cultivated in full N-Medium with and without 20  $\mu$ M CuSO<sub>4</sub>.** Fresh weight was positively correlated with number of fronds in all four treatments after eight days of cultivation.



**Figure 10-6: Anthocyanin concentration of *S. polyrhiza* offspring cultivated in full N-medium with and without 20  $\mu$ M CuSO<sub>4</sub>.** Concentration of anthocyanin was increased in offspring plants grown in CuSO<sub>4</sub> medium as background and actual treatment. Different letters indicate a significant difference ( $P < 0.05$ ) according to a Tukey's honest significance test.  $P$ -values of a two-way ANOVA are shown. FW = fresh weight.

## **11 Declaration of Authorship**

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Jena, 28. Februar 2017

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Saskia Gablenz