

## RESEARCH ARTICLE



# Reduced iron and cobalt levels in response to curcumin supplementation are not responsible for the prolonged larval development and do not affect the oxidative stress tolerance and polyamine status of *D. melanogaster*

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## Abstract

Recent reports indicated that the phytochemical curcumin possesses iron-chelating activity. Here, by employing the fruit fly *Drosophila melanogaster*, we conducted feeding studies supplementing curcumin or, as a control, the iron chelator bathophenanthroline (BPA). First, the absorption and further metabolism of dietary curcuminoids were proved by metabolomics analyses. Next, we found that 0.2% dietary curcumin, similar to BPA, lowered the iron but also the cobalt content, and to a lesser extent affected the manganese and zinc status. Supplementation during larval stages was required and sufficient for both compounds to elicit these alterations in adult animals. However, curcumin-induced retarded larval development was not attributable to the changed trace metal status. In addition, a reduction in the iron content of up to 70% by curcumin or BPA supplementation did not reduce heme-dependent catalase activity and tolerance toward H<sub>2</sub>O<sub>2</sub> in *D. melanogaster*. Moreover, polyamines were not influenced by curcumin treatment and decreased iron levels. This was confirmed for selected organs from 0.2% curcumin-treated mice, except for the spleen. Here, elevated spermidine level and concomitant upregulation of genes involved in polyamine production were associated with a putatively anemia-derived increased spleen mass. Our data underline that the metal-chelating property of curcumin needs to be considered in feeding studies.

**Abbreviations:** AdoMet, S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase; BPA, bathophenanthroline; ConEx assay, consumption-excretion assay; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; ICP MS, inductively coupled plasma mass spectrometry; ODC, ornithine decarboxylase; SAT, spermidine/spermine S-acetyltransferase; SMO, spermine oxidase.

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## KEYWORDS

bathophenanthroline, catalase, cobalt, curcumin, development, *Drosophila melanogaster*, iron, polyamines, trace metals

## 1 | INTRODUCTION

Turmeric powder derived from the rhizome of the plant *Curcuma longa* (turmeric, family Zingiberaceae) is a commonly used spice (curry, mustards) and yellow food dye (E100) that is rich in phenolic compounds, namely in curcuminoids.<sup>1,2</sup> In traditional Indian and Chinese medicine, curcuma has been used for many centuries to support wound healing and treat a variety of ailments such as abdominal disorders, inflammatory diseases, biliary complaints, or hepatic diseases.<sup>3</sup> Numerous in vitro and in vivo studies confirmed the health-beneficial potential of curcumin, which is usually attributed to its antioxidant, anti-inflammatory, and anti-proliferative properties.<sup>1,3</sup> In line with this, curcumin supplementation studies resulted in extended lifespans of model organisms such as fruit flies, nematodes, and mice.<sup>4–8</sup>

The fact that Phase I studies demonstrated virtually no toxicity in humans and curcumin preparations have been “generally recognized as safe” as a food additive at levels up to 20 mg per serving by the FDA facilitated the execution of clinical trials.<sup>2</sup> These studies indicated that curcumin may have therapeutic value against several chronic diseases including neoplastic, neurological, cardiovascular, pulmonary, metabolic, and psychological diseases.<sup>3</sup> A recent scoping review of clinical trials concluded that there is evidence of positive effects of curcumin treatment in particular on diseases associated with inflammation and obesity.<sup>9</sup> However, critics noted that up to now curcumin has never been shown to be conclusively effective in a randomized, placebo-controlled clinical trial for any indication.<sup>2</sup>

Since curcumin is classified as a PAIN compound (pan-assay interference compound), it possesses pleiotropic activity, which makes it extremely difficult to decipher the exact underlying mode of action. The molecular architecture of this phenolic compound allows the interaction with a bunch of proteins, among others, with different receptors, growth factors, kinases, transcription factors, enzymes, adhesion molecules, apoptotic regulators, cytokines, and other proteins.<sup>1,3</sup> On the other hand, curcumin is characterized by a low bioavailability (<1%), since it exhibits a high chemical instability and a low aqueous solubility and is poorly absorbed by intestinal cells and rapidly metabolized by the liver for elimination from an organism.<sup>1,2,10</sup> The majority of orally ingested curcumin is excreted unchanged in the feces.<sup>11</sup>

Accordingly, studies in animal models and humans revealed that it is rather unlikely that substantial concentrations of curcumin can be achieved in the body.<sup>12,13</sup> Hence, it is questionable whether all of the myriad curcumin effects seen in in vitro studies are transferrable to the organismic level of animals and humans. In recent years, there has been increasing evidence that curcumin supplementation positively influences gastrointestinal health and function, including the composition of the microbiota. Since most of the adverse pharmacokinetic parameters of curcumin are not relevant here, it has been suggested that the gastrointestinal tract may be the main region of action and account for many of medicinal benefits of curcumin.<sup>10</sup> Of note, some recent human case studies reported that new pharmaceutical formulations which enhance the bioavailability of curcuminoids<sup>14</sup> were associated with hepatotoxicity or autoimmune hepatitis.<sup>15,16</sup> Similar findings have been reported for other plant bioactives, especially when their bioavailability has been increased by pharmacological means.<sup>17,18</sup> Secondary plant compounds are usually recognized by the body as xenobiotics and, hence, induce the Nrf2-dependent detoxification pathway. Accordingly, one can assume that too high an intake of these compounds can no longer be processed by the liver.

Iron is an essential micronutrient that is required in numerous biological processes including oxygen transport, cellular respiration, gene regulation and DNA biosynthesis. It is a cofactor for heme-containing and iron-sulfur cluster proteins. However, since iron is a redox-active metal, free iron can react with peroxides generating reactive oxygen species.<sup>19–21</sup> Hence, iron homeostasis is crucial for health and low dietary iron causes anemia, while iron overload is toxic and leads to organ dysfunction and correlates with diseases such as liver cirrhosis and hepatoma.<sup>19,20</sup> In previous studies, Jiao et al.<sup>22,23</sup> and our group<sup>24</sup> reported that curcumin supplementation affects the iron metabolism and the iron status of mice. Feeding 0.2% curcumin for 6 months led to significantly reduced iron levels in the liver and spleen of female C57BL/6J mice without affecting zinc and copper levels in these tissues.<sup>24</sup> In a follow-up study by Jiao et al.,<sup>23</sup> curcumin elicits a dose-dependent decline of the iron-related hematologic parameters hematocrit, hemoglobin, serum iron, and transferrin saturation in C3H mice that were maintained under conditions of subclinical iron deficiency. Recently, Samba-Mondonga et al.<sup>25</sup> showed

that curcumin supplementation caused mild anemia and lowered iron stores in a DSS induced colitis mouse model. Taken together, these data are in good agreement with the fact that the 1,3-dicarbonyl moiety of curcumin is an excellent iron chelator.<sup>26</sup>

Remarkably, both curcumin supplementation and iron depletion have been shown to lower the polyamine content in cancer cell lines by affecting key enzymes of polyamine metabolism.<sup>27–29</sup> Polyamines are aliphatic polycations involved in numerous biological processes such as proliferation, differentiation, development, stress response, and aging.<sup>30–32</sup> Polyamine synthesis requires the amino acid ornithine and the amino acid metabolite *S*-adenosylmethionine (AdoMet). Both precursors are decarboxylated by the rate-limiting enzymes ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC), respectively. The products, the diamine putrescine and decarboxylated AdoMet (the latter representing a donor of an aminopropyl moiety) are substrates of the spermidine synthase. Spermidine is elongated to spermine by the addition of a second aminopropyl moiety catalyzed by spermine synthase. The spermidine pool can also be replenished from spermine by spermine oxidase (SMO) activity.<sup>33</sup>

In the fruit fly *Drosophila melanogaster* many aspects of iron homeostasis and polyamine metabolism are conserved with respect to the situation in mammals.<sup>21,34,35</sup> However, it must be borne in mind that mammals have an extraordinarily high iron requirement for erythropoiesis due to their hemoglobin-dependent oxygen transport system, which is far above the iron demands of all other physiological areas, and which is not reflected in insects with their trachea-based respiratory system.<sup>21</sup> In the present study, we employed *D. melanogaster* to investigate the impact of curcumin feeding compared to supplementing the diet with the established iron chelator bathophenanthroline (BPA) on development, mineral status, and polyamine pattern. In addition, we analyzed the impact of curcumin supplementation on the polyamine levels and gene expression of polyamine key enzymes in mice tissues derived from the abovementioned former curcumin feeding study.<sup>24</sup>

## 2 | EXPERIMENTAL PROCEDURE

### 2.1 | Fly husbandry and feeding protocols

The *D. melanogaster* wild-type strain Oregon-R (Bloomington stock center) was used. Animals were cultured on Caltech medium (3% sucrose, 5.5% glucose, 6%

corn meal, 2.5% yeast extract, 1% agar, 0.03% tegosept, and 0.3% propionic acid) at 25°C and 60% humidity with a 12 h day-night cycle. For all experiments, flies were synchronized by collecting eggs on grape plates at 25°C. Experimental diets were supplemented with 0.2% or 0.5% curcumin (Carl Roth, Karlsruhe, Germany; 98% pure standard) or 100 µM of the iron chelator bathophenanthrolinedisulfonic acid disodium salt (BPA; Sigma-Aldrich, Taufkirchen, Germany). Since the curcumin stock was solved in ethanol, all experimental media contained 2% ethanol.

To study the impact of curcumin and BPA supplementation on fruit flies, different feeding protocols were applied. Supplements (curcumin, BPA) were given either during larval (L-feeding) or adult (A-feeding) stages only or during the entire experimental period (E-feeding; see Figure S1). Adult flies were separated by sex on day 3 and transferred to fresh medium every second day.

### 2.2 | Egg to adult development

Twenty eggs per vial were placed on the different experimental diets and the egg to adult development was monitored by daily twice inspection. The experiment was repeated three times.

### 2.3 | Larval feeding assay

Larval food intake analysis was carried out according to Kaun et al.<sup>36</sup> with modifications. Female fruit flies were allowed to lay eggs for 8 h on standard CT medium and the synchronized eggs were cultured under standard conditions. At day 4, the third instar larvae were placed on Petri dishes containing the different experimental diets (control, 0.2% curcumin, 0.5% curcumin or 100 µmol BPA) for 2 h. Subsequently, the larvae were transferred to the same medium stained with 0.2% Erioglaucine dye (aka FD&C Blue No. 1, Sigma-Aldrich). After 60 min at 25°C, the Petri dishes were placed on ice. The larvae were washed in PBS, collected in a 2 mL reaction tube and lysed in 500 µL dH<sub>2</sub>O and 500 µL canola oil by using a TissueLyser (QIAGEN, Hilden, Germany). Following phase separation by centrifugation at 4°C and 10,000×g for 3 min, the water-soluble blue dye was in the aqueous layer, while curcumin accumulated in the organic oil fraction. 75 µL of the lower aqueous phase were mixed with 75 µL dH<sub>2</sub>O and measured at 620 nm in 96 well plates (Microplate Reader; Labsystems Oy, Helsinki, Finland) to calculate the amount of ingested food.

## 2.4 | Consumption-excretion (ConEx) assay for food intake of adult flies

Feed intake of adult flies was determined by the ConEx assay described in (Wu, 2019). Briefly, age-matched male or female flies were raised on control diet or according to the abovementioned E-feeding protocol. On day 10, flies were transferred to vials that were prepared according to (Wu, 2019). The microtube cap was filled with either control medium or medium supplemented with 0.2% curcumin or 100  $\mu$ M BPA. In addition, all media contained 0.5% of the blue dye Erioglaucine. After 24 h, the flies were removed and the blue dye that had been excreted in fecal spots was collected by washing with 1 mL water. Samples were extracted with 200  $\mu$ L diethylether to eliminate lipophilic chromatophores (in particular curcumin), before the absorbance at 620 nm was determined.

## 2.5 | Sample preparation for Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS)

Starting with eggs, fruit flies were fed a control diet or experimental diets that were supplemented with 0.2% curcumin and 100  $\mu$ M BPA, respectively, according to the E-feeding protocol. On day 3 after eclosion, the adult flies were sorted by sex and females were transferred in groups of 20 animals into vials containing the respective control or experimental diet. For the remaining seven days, the animals were placed on fresh medium every second day. On day 10, the female flies were starved on 2% agar for 2 h before they were shock frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Simultaneous metabolite, protein, lipid extraction (SIMPLEX) was carried out as described previously.<sup>37</sup> Briefly, a methanol/ultra-pure water mixture (1:1, v/v) was added to the samples (10  $\mu$ L/mg fly wet weight) and flies were lysed for 4.5 min at 25 Hz; 100  $\mu$ L of the homogenate were pipetted into 15 mL falcon tubes (Sarstedt, Nuernbrecht, Germany) followed by 500  $\mu$ L methanol and 4 mL methyl-tert-butylether. After incubation in an end-over-end rotator (25 rpm) for 30 min at room temperature, 500  $\mu$ L ultra-pure water was added. The samples were mixed, incubated for 10 min at room temperature and centrifuged at  $4^{\circ}\text{C}$  for 10 min at  $1000\times g$ . The upper phase containing the lipophilic metabolites was transferred to a new falcon tube. The remaining sample was extracted a second time repeating the extraction steps described above. Lipophilic and hydrophilic phases were evaporated to dryness before being resuspended in 500  $\mu$ L isopropanol/chloroform (3:1, v/v; lipophilic metabolites) and 500  $\mu$ L methanol/

ultra-pure water (1:1, v/v; hydrophilic metabolites), respectively. Samples were stored at  $-80^{\circ}\text{C}$ . For the FT-ICR-MS analysis all samples were diluted 1:100. The experiment was conducted three times. For each sample three technical replicates were analyzed.

## 2.6 | FT-ICR-MS and data analyses

Samples were placed in an autosampler (temperature  $4^{\circ}\text{C}$ ) and analyzed with an extreme-high-resolution Fourier transform—ion cyclotron resonance—mass spectrometer (solari-XR, Bruker, Bremen, Germany). The mass spec was linked with an uHPLC system (Agilent 1260 Infinity HPLC, Waldbron, Germany) in the direct injection mode (that means without column separation). As eluent, methanol/ultra-pure-water (1:1, v/v) was also used with a flow of 10  $\mu$ L/min. Samples were ionized with an electro-spray-ionization source in both modes. Data were recorded with 4 M data mode. Therefore, 200 spectra were acquired and averaged. Additional method parameters of FT-ICR-MS are listed in Table S1. All methods were calibrated by sodium tri-fluor acetic acid (NA-TFA) with a precision of <500 ppb.

After analyzing, all data were re-calibrated with matrix component. Therefore, a customized local calibration list with >60 typical matrix molecules were used. All data were recalibrated with a valued <100 ppb. Peak annotation was conducted with MetaboScape 2021b (Bruker, Bremen, Germany) with local databases, which based on metabolites out of the KEGG database as well as local customized databases based on a literature search (e.g., for the different curcuminoids, curcumin derivatives and curcumin degradation products; see Table S2). The raw bucket tables (annotated metabolites) were exported and further processed in R (version 4.1.2). Data were processed (e.g., data merging) in accordance with Brix et al.<sup>38</sup>

Metabolites were not further analyzed if at least two of the three technical replicates of each sample showed no detectable signals. In a next step, compounds that specifically appeared only in the curcumin and BPA treatment group samples, respectively, were copied to a separate list, which contains putative degradation products and impurities of the additives. Then, all missing values were replaced by knn sample-wise values, before the data set was normalized and further analyzed by MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>) for metabolites that were changed at least 1.2-fold in response to curcumin or BPA treatment with a FDR-corrected statistical significance value of  $p \leq 0.1$  (one-way Anova).



## 2.7 | Trace metal determination

For each sample, 110 ten day old adult flies that had undergone the feeding protocols E, L, and A, respectively, were harvested after a fasting period of 2 h to avoid trace metal contamination from ingested food. Their body weights (BW) were determined, before selected trace metals were analyzed by inductively coupled plasma mass spectrometry (ICP MS; SYNLAB, Jena, Germany). The experiment was repeated three times.

## 2.8 | Catalase assay

Catalase activity was determined spectrophotometrically following the protocol of Mackay and Bewley.<sup>39</sup> Ten day old adult flies that had undergone the different feeding protocols were harvested. For each sample, 10 flies were weighed and subsequently lysed in 500  $\mu$ L 20 mM HEPES buffer (pH 7.0), 0.1% Triton X-100 for 6 min in a TissueLyser. After centrifugation at 4°C for 20 min, 100  $\mu$ L of the supernatant was added to 800  $\mu$ L of 50 mM phosphate buffer (pH 7.0) in a quartz cuvette. The catalase reaction was started by the addition of 100  $\mu$ L of 19.6 mM  $H_2O_2$  solution and the decline of absorbance was immediately monitored at 240 nm for 2 min (DU 800 spectrophotometer, Beckman Coulter, Krefeld, Germany). Using a molar extinction coefficient for  $H_2O_2$  of 62.4, catalase activity was calculated according to Lubinsky and Bewley.<sup>40</sup>

## 2.9 | Oxidative stress resistance assay

Flies were raised following the E-, A-, or L-feeding protocol and sorted by sex on day 3 after eclosion, before being placed in groups of 20 flies in vials containing control diet. For the remaining seven days, they were placed on fresh medium every second day. On day 10, the flies were starved for 2 h and then transferred to new vials containing a filter soaked with either a 5% sucrose solution only or a 5% sucrose solution with 10%  $H_2O_2$  as described previously.<sup>41</sup> The survival rates of the flies were monitored over time. The experiment was repeated three times.

## 2.10 | Polyamine determination by HPLC

The polyamine level of fruit flies and murine tissues were determined according to Esatbeyoglu et al. and Heinick et al.<sup>42,43</sup> Murine tissues derived from a former curcumin feeding study.<sup>24</sup> For fruit fly studies, 10 day old adult animals that had undergone the different feeding protocols were harvested after a fasting period of 2 h to avoid polyamine

contamination from ingested food. 10–20 mg of murine tissue or 15 flies per sample were weighed and lysed in 1 mL 5% TCA containing 2  $\mu$ g/mL diaminohexane (internal standard) in the TissueLyser (10 min at 25 Hz). After centrifugation at 10,000 $\times$ g for 20 min at 4°C, 500  $\mu$ L of the supernatant were mixed with 250  $\mu$ L of saturated sodium bicarbonate solution along with a small sparing tip of  $NaHCO_3$  powder. 500  $\mu$ L dansyl chloride (7.5 mg/mL acetone) was added and, subsequently, the samples were incubated in the dark at 37°C for 1 h, before 250  $\mu$ L 100 mg/mL L-proline were added. Following an additional incubation for 20 min, dansylated polyamines were extracted by 900  $\mu$ L ethyl acetate. The solvent was evaporated and the pellet was resuspended in 100  $\mu$ L acetonitrile. 20  $\mu$ L of each sample was separated by reversed phase chromatography on a nucleosil 5  $\mu$ m  $C_{18}$  column (250  $\times$  4.6 mm, Phenomenex) at a flow rate of 0.8 mL/min applying the following gradient with mobile phase A water and mobile phase B acetonitrile: 0 min 65% B, 16 min 100% B, and 20 min 100% B. In order to record the complete polyamine spectrum, dansylated polyamines were detected by UV absorption at  $\lambda = 254$  nm (DAD, Jasco) and simultaneously by more sensitive fluorescence detection (FD, Jasco; excitation wavelength 365 nm, emission wavelength 480 nm; signals were amplified 100-fold).

## 2.11 | Quantitative real time PCR

Total RNA was isolated with TriFast reagent (VWR, Darmstadt, Germany) from murine tissues derived from a former curcumin feeding study.<sup>24</sup> Quantitative real time PCR was carried out according to Piegholdt et al.<sup>41</sup> The primer pairs are listed in Table S3.

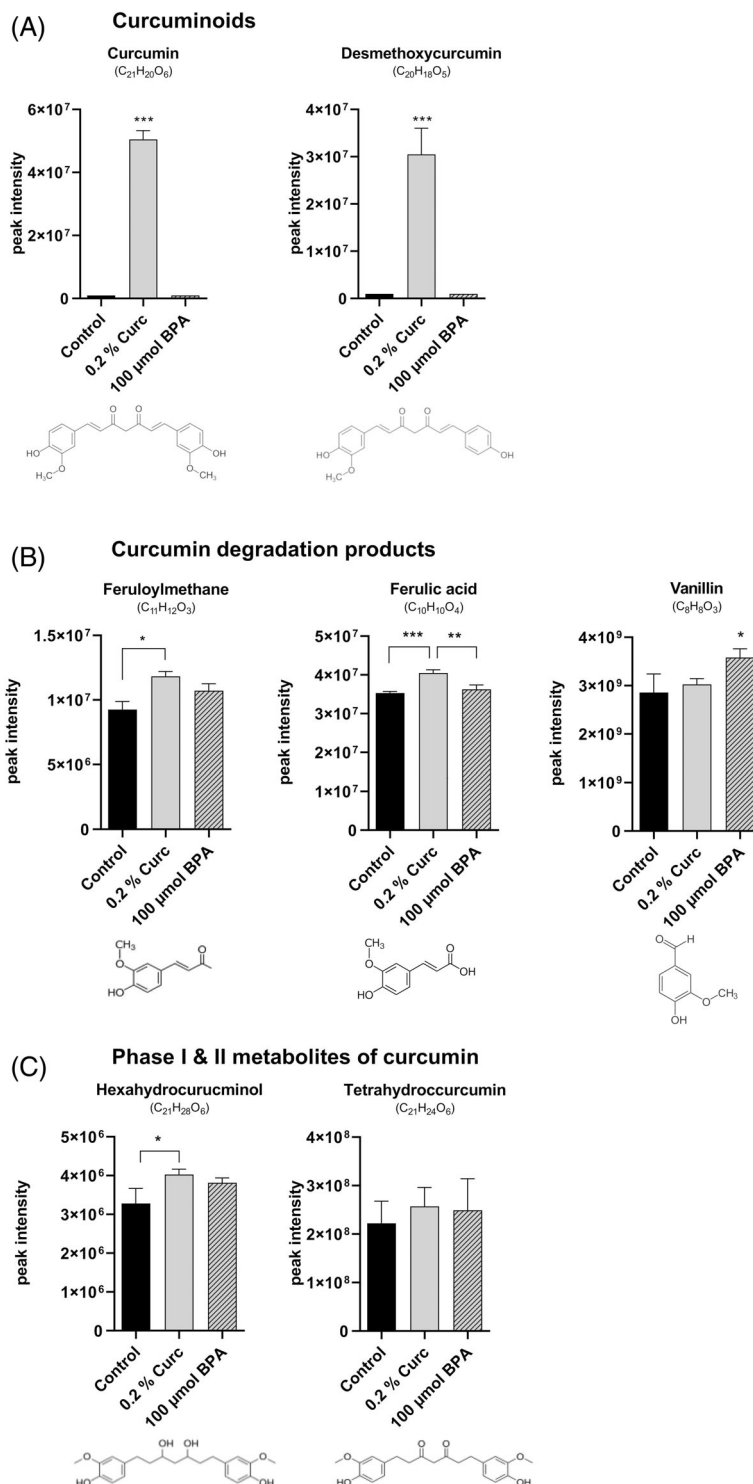
## 2.12 | Statistical analysis

For statistical analyses, GraphPad Prism 9 was used. The results are expressed as means  $\pm$  standard deviation. Before analyses, all data were tested for normality of distribution (Shapiro-Wilk tests). If the data were normally distributed, a two-sided Student's *t*-test was performed. Otherwise, the nonparametric Mann–Whitney U test was used for not normally distributed data.

# 3 | RESULTS

## 3.1 | Curcuminoids and their degradation products are found in curcumin-supplemented female fruit flies

To elucidate whether fruit flies take up and further metabolize supplemented curcuminoids, an FT-ICR-MS analyses



**FIGURE 1** Curcuminoids and their degradation products are present in *D. melanogaster* females fed a curcumin-supplemented diet. Following the E-feeding protocol (starting treatment from the eggs laid), *D. melanogaster* females were fed a control and a 0.2% curcumin-supplemented diet, respectively. On day 10 after eclosion, adult female fruit flies were harvested and lysed. The metabolites were analyzed by FT-ICRMS and annotated via their *m/z* values using the compounds listed in Table S2 as reference. Data represent the means  $\pm$  SD of three independent experiments ( $n = 60$  flies per experiment and treatment) measured in triple replications (one-way ANOVA (Dunnnett's multiple comparisons test);  $p$ -value = \*0.05; \*\*0.01; \*\*\*0.001).

approach was chosen. To this end, we first confirmed the presence of the three regular curcuminoids curcumin ( $C_{21}H_{20}O_6$ ), demethoxycurcumin ( $C_{20}H_{18}O_5$ ), and bide-methoxycurcumin ( $C_{19}H_{16}O_4$ )<sup>1,44</sup> in our curcumin standard (data not shown). Next, we employed female fruit flies reared according to the E-feeding protocol (i.e., starting with eggs until the adult stage at the age of 10 days) and considered curcuminoids and their derivatives and degradation products known from former laboratory rodent and human studies, summarized in Table S2.<sup>1,12,45</sup> Of the three curcuminoids of the standard, compounds with molecular masses corresponding to curcumin and demethoxycurcumin were identified in 0.2% curcumin supplemented animals (Figure 1A). Compounds with molecular masses of the curcumin degradation products vanillin, ferulic acid, and feruloyl methane were already present in fruit flies fed a control diet. This is not surprising, since they could derive also from the metabolism of other phenolic compounds present in the *Drosophila* food. However, the relative amounts of ferulic acid and feruloyl methane were significantly elevated solely in curcumin supplemented fruit flies (Figure 1B), whereas the vanillin signal was not altered by curcumin supplementation. We then looked for compounds reported as curcumin detoxification products of phase I and phase II metabolism in laboratory rodents and humans (Table S2). Again, we identified molecules with masses corresponding to tetrahydrocurcumin ( $C_{21}H_{24}O_6$ ), octahydrocurcumin/hexahydrocurcuminol ( $C_{21}H_{28}O_6$ ) already in the control diet group, of which only the latter metabolite was significantly enhanced by curcumin feeding (Figure 1C). Overall, our data indicate that curcumin uptake and further metabolism occurred at a detectable level in fruit flies.

### 3.2 | Curcumin supplementation altered the trace metal pattern of *D. melanogaster*

Supplementing the *D. melanogaster* diet with 0.2% or 0.5% curcumin following the E-feeding protocol (i.e., starting supplementation of the diet at the egg stage until day 10 of adulthood) affected the trace metal profile of female and male fruit flies. The iron content was significantly reduced by 0.2% curcumin from  $41.7 \pm 5.6$  ng/mg wet weight to  $31.8 \pm 2.6$  ng/mg wet weight for females (Figure 2A) and from  $41.9 \pm 3.4$  ng/mg wet weight to  $32.6 \pm 1.8$  ng/mg wet weight for males (Figure 3A). A higher curcumin dose of 0.5% did not significantly further increase this effect. Apart from this, curcumin supplementation markedly decreased the cobalt levels in female and male flies by 40% and 50%, respectively (Figures 2D and 3D). Moreover, curcumin had a sex-specific effect on the zinc content of fruit flies. In males, the zinc level was increased from  $39.0 \pm 0.4$  ng/mg wet weight to  $50.7 \pm 2.8$  ng/mg wet weight (Figure 3G),

whereas the zinc content of females remained almost unchanged (Figure 2G). Again, increasing the curcumin concentration in the diet to 0.5% had only a minor additional effect on the status of this metal ion. Manganese levels were also slightly but not significantly decreased by curcumin treatment in both sexes (Figures 2J and 3J). The contents of other metal ions, namely magnesium, calcium, copper, molybdenum, selenium, and chrome were not affected by curcumin in female and male fruit flies (Figures S1 and S2).

As expected, supplementing 100  $\mu$ M of the iron chelator BPA had a strong effect leading to an approximately 70% reduction of the iron content in female and male flies (Figures 2A and 3A). Remarkably, similar to curcumin, BPA supplementation also caused a significant reduction of the cobalt concentrations in females and males (Figures 2D and 3D). Moreover, manganese levels were also slightly reduced in females and more evidently in males (Figures 2G and 3G). However, in contrast to curcumin treatment, the zinc and copper contents of male and female flies were decreased by supplementing BPA (Figures 2J, 3J, and S2), while magnesium and calcium were slightly elevated solely in males (Figures S1 and S2).

Taken together, curcumin and also BPA supplementation during the entire development until day 10 of adulthood did not only lowered the iron status of *D. melanogaster*, but additionally led to alterations of the cobalt, manganese, and zinc content.

### 3.3 | Curcumin supplementation resulted in a delayed egg to pupae development

Raising *D. melanogaster* on a diet supplemented with 0.2% or 0.5% curcumin resulted in a delayed egg to pupae development (Table 1) whereas the pupae to adult developmental time of 4.0 days remained unchanged when compared to flies fed control medium. This was accompanied by an about 50% reduced feed intake of *D. melanogaster* larvae (Figure 4A). Yet, the applied curcumin concentrations were not toxic for developing fruit flies indicated by the similar survival rate (i.e., the percentage of eclosed adults related to the number of eggs). Since treatment with 100  $\mu$ M BPA did not affect the developmental time, we conclude that a reduced iron content was not responsible for the curcumin-induced retarded development. The prolonged feeding period of curcumin-treated larvae appears to compensate the lower intake rate, as the BW and body composition of pre-pupal wandering instar 3 larvae (which no longer ingest food) were similar to those of control animals (Table 1). In contrast to the situation in larvae, curcumin supplementation did not affect the food intake in adult female and male flies (Figure 4B,C).

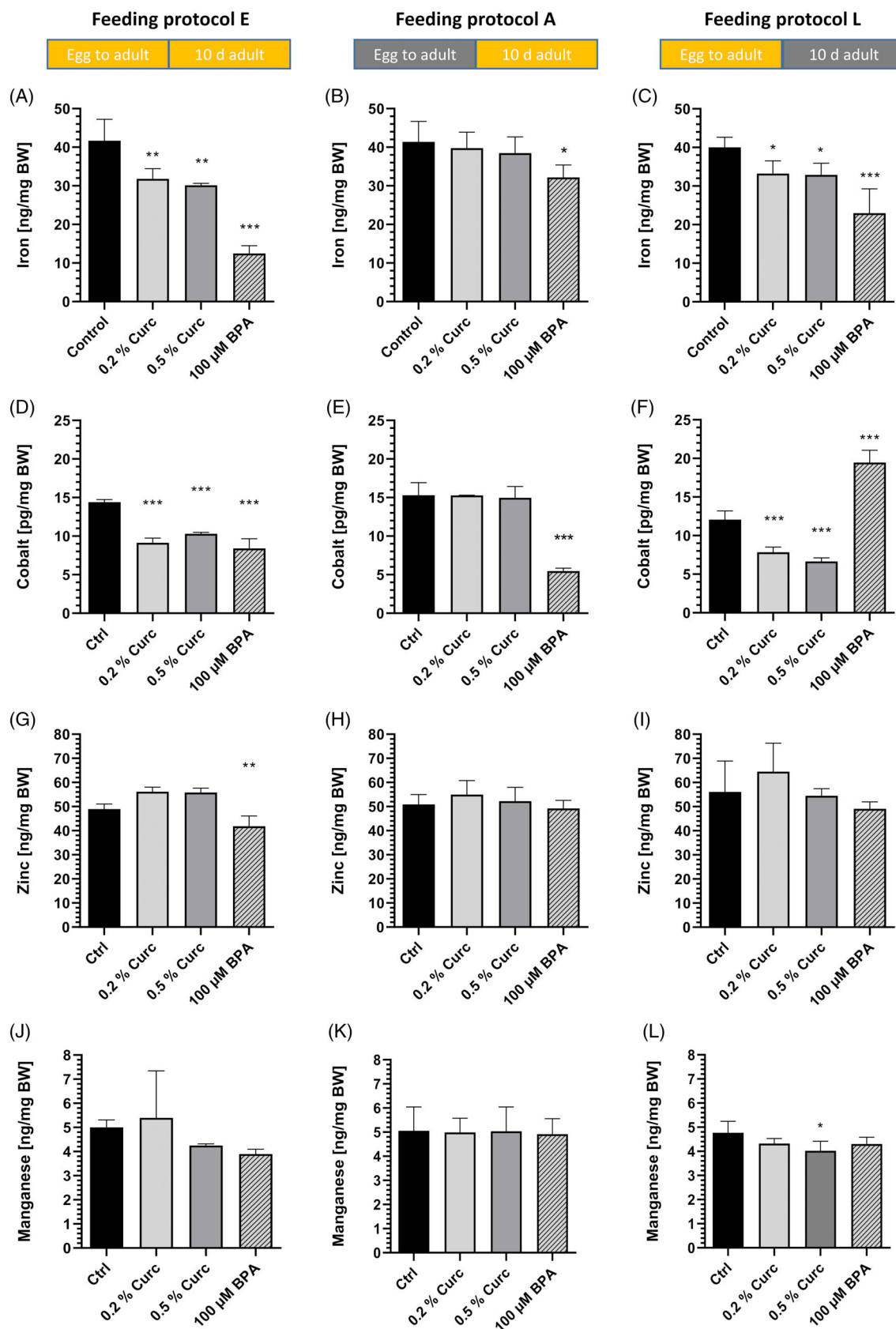


FIGURE 2 Legend on next page.



Moreover, their BWs were not altered by supplements when applying the A- and L-feeding protocols (Table 2) except for males treated with 0.5% curcumin during larval development. The most pronounced reduction in BW compared to the control group was caused by the E-feeding protocol, but remarkably exclusively in females, reaching statistical significance for fruit flies exposed to 0.5% curcumin and 100  $\mu$ M BPA, respectively (Table 2).

### 3.4 | Alterations in trace metal pattern required larval feeding of curcumin

We next asked whether feeding *D. melanogaster* a diet supplemented with curcumin exclusively during the adult stage starting immediately after eclosion until the age of 10 days (A-feeding protocol) changes the iron content and the trace metal pattern of fruit flies in a similar manner as the E-feeding protocol. As shown in Figures 2B and 3B, supplementation of 0.2% or 0.5% curcumin following the A-feeding protocol did not significantly affect the content of iron and the pattern of any of the examined trace metals in female and male fruit flies, except for calcium, which was slightly but significantly elevated in the female 0.5% curcumin treatment group with  $119.0 \pm 6.0$  ng/mg BW versus  $106.7 \pm 5.4$  ng/mg for controls (Figures 2E,H,K, 3E,H,K, S1 and S2).

The influence of the iron chelator BPA on the iron content of fruit flies was also lowered when the exposure takes place solely during the adult stage. For females but not for males the iron content was still significantly reduced by 25% (Figures 2B and 3B). In contrast, the impact of a BPA treatment that was restricted to the adult stage on the cobalt content in males and females were similar to the effects obtained by the E-feeding protocol (Figures 2E and 3E). The pattern of the other trace metals examined remained unchanged by BPA when the A-feeding protocol was applied (Figures 2E,H,K, 3E,H,K, S1, and S2).

### 3.5 | Alterations in trace metal pattern owing to larval feeding of curcumin cannot be reversed in adult flies

Since the alterations of the metal ion pattern obtained by the E-feeding protocol of curcumin cannot be

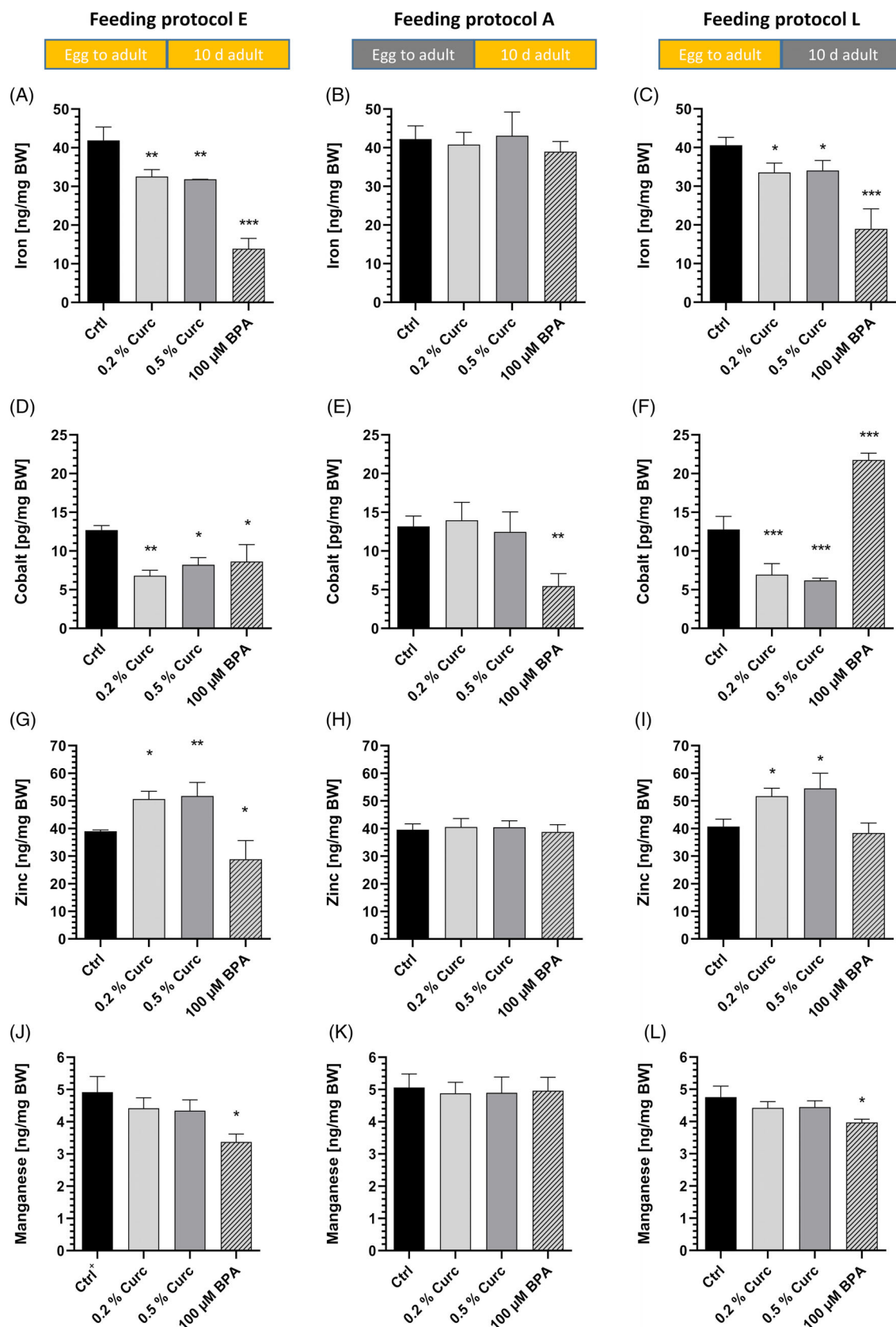
explained by the feeding activity during the adult stage, we next investigated whether they can be elicited by feeding curcumin exclusively during larval development. By applying feeding protocol L, we found that supplementation of curcumin solely during the larval stages is sufficient to cause similar alterations as those found for flies that were fed according to the E-feeding protocol. 0.2% curcumin significantly decreased the iron content in females and males by about 25% and 20% (Figures 2C and 3C), and the cobalt levels by about 40% and 50%, respectively (Figures 2F and 3F). Again, curcumin led to slightly augmented zinc levels in male flies (Figure 3I). These data indicate that although curcumin was applied only during larval development and the eclosed adult flies were fed for 10 days on control diet, males and females were not able to reverse the changes in the iron, cobalt, and zinc pattern that were implemented by curcumin during larval feeding.

In good agreement with the curcumin data, a BPA treatment that was restricted to the larval stages lowered the iron content of both male and female flies in a similar way as the E-feeding protocol (Figures 2C and 3C). In contrast to that, the effect of larval BPA supplementation on the cobalt status in adult fruit flies was strikingly different when compared to the curcumin results and to the data obtained for the E-feeding protocol for BPA. The cobalt levels of both, males and females were not decreased but significantly elevated by larval BPA treatment (Figures 2F and 3F).

### 3.6 | Curcumin and BPA supplementation did not change the cobalamin marker methylmalonic acid but may led to a reduction in porphyrin synthesis in *D. melanogaster*

Cobalt is a component of vitamin B<sub>12</sub> (cobalamin), which functions as a coenzyme in methionine synthase and methylmalonyl-CoA mutase reactions. Although both curcumin and BPA supplementation reduced the cobalt level in *D. melanogaster* females, our FT-ICR-MS analysis revealed that the metabolite that corresponds to the molecular mass of methylmalonic acid (C<sub>4</sub>H<sub>6</sub>O<sub>4</sub>), a cobalamin marker in mammals, was not changed (Figure 5A).

**FIGURE 2** The iron, cobalt, zinc, and manganese levels of female *D. melanogaster* are affected by curcumin and bathophenanthroline (BPA) supplementation, respectively. Fruit flies were cultivated according to the feeding protocols E, A, or L (yellow bars indicate periods on experimental curcumin- or BPA-supplemented diets, gray bars periods on control diet). On day 10 of adulthood, animals were harvested and the trace metal content of females were determined by ICP-MS. Data represented the means  $\pm$  standard deviation of three independent replicates with  $N = 100$  flies per concentration and repetition (one-way ANOVA with Dunnett's multiple comparisons test;  $p$ -value = \*0.05; \*\*0.01; \*\*\*0.001).

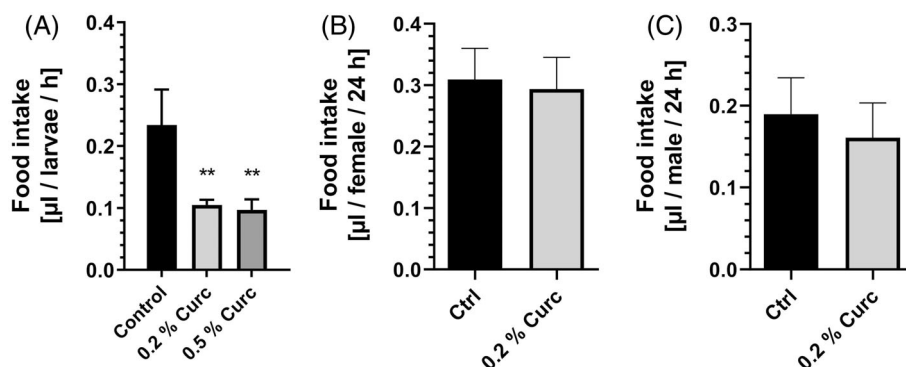


**FIGURE 3** The iron, cobalt, zinc, and manganese levels of male *D. melanogaster* are affected by curcumin and bathophenanthroline (BPA) supplementation, respectively. Male fruit flies were treated as described for females in Figure 1. Data represented the mean trace metal contents  $\pm$  standard deviation of 10 day old males determined by ICP-MS (three independent replicates with  $N = 100$  flies per concentration and repetition; one-way ANOVA with Dunnett's multiple comparisons test;  $p$ -value = \*0.05; \*\*0.01; \*\*\*0.001).

**TABLE 1** Curcumin treatment prolongs the time required for the egg to pupae development in *D. melanogaster* without effecting the body weight (BW) and body composition of wandering third instar larvae.

	Egg to pupae time (d)	BW third instar (mg)	BC third instar TAG/protein	Egg to adult (%)
Control	6.0 ± 0.2	1.83 ± 0.15	1.249 ± 0.022	87.8 ± 2.5
0.2% Curcumin	6.6 ± 0.5***	1.91 ± 0.17	1.252 ± 0.023	81.6 ± 5.8
0.5% Curcumin	6.9 ± 0.3***	1.91 ± 0.24	1.238 ± 0.018	86.1 ± 5.1
100 µM BPA	6.0 ± 0.1	1.90 ± 0.17	1.241 ± 0.025	81.7 ± 6.0

Note: Eggs were placed on CT medium supplemented with the indicated curcumin or BPA concentrations. Fruit fly development was monitored over time by daily counting the number of emerged pupae and eclosed flies, respectively. Data for development times and ratios represent the means of three independent experiments performed in triplicate with  $N = 180$  animals per condition (one-way ANOVA (non-parametric Kruskal-Wallis test) with Dunn's multiple comparisons correction (\*\* $p < 0.001$ ). The results for BW and body composition (BC) of third instar larvae represent the means ± SD of three independent experiments performed in triplicate with  $N = 90$  animals per condition. One way ANOVA with Dunnett's multiple comparisons correction.



**FIGURE 4** Curcumin-treatment reduced the food intake of *D. melanogaster* larvae but not of adults. (A) *D. melanogaster* third instar larvae that had been reared on control medium followed by a 2 h adaptation on the different experimental media were transferred onto the respective experimental media containing 1% brilliant blue dye. After 1 h of feeding, the larvae were harvested, washed in PBS and lysed. The amount of ingested dye was determined spectrophotometrically in order to calculate the food uptake relative to the control. Data represent the means ± standard deviation of three independent determinations performed in duplicate with  $N = 120$  animals per condition (one-way ANOVA, \*\* $p < 0.01$ ). (B) Age-matched female or (C) male fruit flies were raised on control or curcumin-supplemented diet according to the E-feeding protocol (starting treatment from the eggs laid). On day 10, adult flies were transferred to ConEx-vials (see material and methods) containing erioglaucin-dyed experimental media. The animals were cultured for an additional 24 h, before the excreta were collected. The amount of excreted dye was determined spectrophotometrically in order to calculate the food uptake. Data represent the means ± standard deviation of three independent quintuple determinations with  $N = 300$  animals per condition.

The other common cobalamin marker homocysteine ( $C_4H_9NO_2S$ ) was not detectable.

Furthermore, since iron serves as the central atom in heme, we were interested whether curcumin and BPA-treatment altered metabolites involved in porphyrin biosynthesis and heme degradation products. In female fruit flies, we detected aminolevulinic acid ( $C_5H_9NO_3$ ), the first compound in the porphyrin synthesis pathway and proporphyrinogen ( $C_{20}H_{20}N_4$ ). Both aminolevulinic acid and proporphyrinogen were reduced in the curcumin and the BPA group, although in any case this did not reach statistical significance (Figure 5B,C). A metabolite with the molecular mass of the ion-free by-product of heme synthesis coporphyrin I ( $C_{36}H_{38}N_4O_8$ ) was identified in all three biological replicates of BPA fruit flies (5 out of 9 analyses), however only in 1 and 2 out of 9 analyses of the control and the curcumin group. Tentative L-

urobilinogen ( $C_{33}H_{48}N_4O_6$ ), a heme degradation product, was detectable only in one out of three biological replicate of the control, however, in two and all three biological replicates of the curcumin and BPA-treated flies, respectively. Moreover, tentative biliverdin IX ( $C_{33}H_{32}N_4O_6$ ) was not regularly identified in the different groups.

### 3.7 | A reduced iron content due to curcumin or BPA feeding did not affect catalase activity and oxidative stress tolerance in fruit flies

To analyze whether the reduced iron level elicited by curcumin or BPA supplementation affects the activity of iron-dependent enzymes, we tested exemplarily the

heme-depending catalase activity in 10 day old male and female adult flies that had undergone the E-feeding, L-feeding, and A-feeding protocol. As shown in Figure 6A–F, in all cases the enzyme activities of treated flies remained unchanged when compared to flies maintained on control medium. Hence, the reduced iron content due to curcumin or BPA supplementation did not affect iron-dependent  $H_2O_2$  detoxifying catalase activity in male and female fruit flies. In good accordance with that, we did not find a negative impact on the stress tolerance of adult male and female fruit flies toward  $H_2O_2$  when the iron content of the animals were reduced

owing to curcumin or BPA feeding (Figure 6G–I). Remarkably, even a slightly increased tolerance to  $H_2O_2$  was observed in BPA-treated flies that contained the lowest iron content of all treatment groups.

### 3.8 | Curcumin supplementation did not affect polyamine levels in *D. melanogaster*

Curcumin treatment and reduced iron levels have been reported to change the polyamine pattern in cell culture studies.<sup>27–29</sup> Therefore, we next determined the

	BW (mg)		
	E-feeding	A-feeding	L-feeding
Females			
Control	1.062 ± 0.080	1.056 ± 0.123	1.046 ± 0.066
0.2% Curcumin	1.019 ± 0.081	1.046 ± 0.113	1.087 ± 0.073
0.5% Curcumin	0.967 ± 0.069***	1.054 ± 0.127	1.027 ± 0.056
100 µM BPA	0.983 ± 0.078**	1.070 ± 0.125	1.066 ± 0.083
Males			
Control	0.666 ± 0.042	0.684 ± 0.038	0.678 ± 0.031
0.2% Curcumin	0.658 ± 0.041	0.676 ± 0.039	0.673 ± 0.031
0.5% Curcumin	0.640 ± 0.040	0.690 ± 0.046	0.646 ± 0.033**
100 µM BPA	0.643 ± 0.040	0.689 ± 0.042	0.664 ± 0.043

TABLE 2 The impact of different curcumin- and BPA-feeding protocols on the body weight (BW) of male and female *D. melanogaster*.

Note: Fruit flies were fed according to the E-, A-, and L-feeding protocol, before their BW was determined on day 10 after eclosion. Data are expressed as means ± standard deviation of 22–28 independent experiments with  $N = 220$ –280 animals per treatment group (\* $p < 0.05$ ).

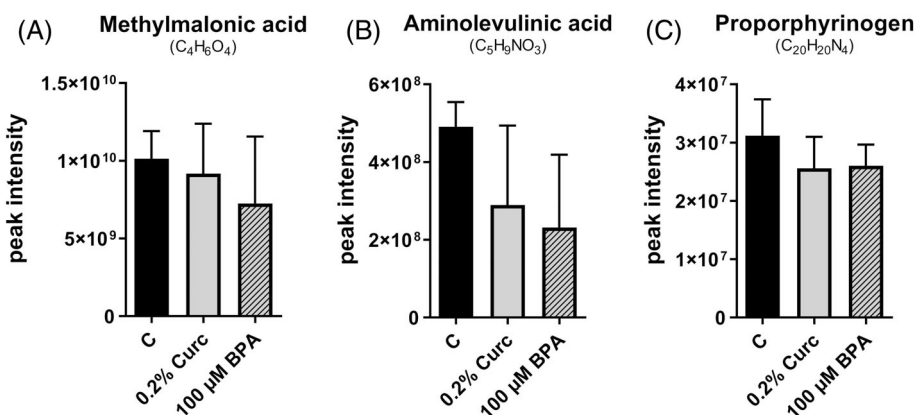


FIGURE 5 Curcumin or BPA treatment did not alter the content of the cobalamin marker methylmalonic acid but led to a reduction of porphyrin/heme synthesis metabolites in *D. melanogaster*. Female fruit flies were reared according to the E-feeding protocol, before they were harvested for FT-ICR-MS analyses. (A) The cobalamin marker methylmalonic acid was not affected by 0.2% curcumin and 100 µM BPA, while (B,C) the peak intensities of the porphyrin precursor metabolite aminolevulinic acid and proporphyrinogen were reduced in both treatment groups, although not statistically significant (one way ANOVA). Data are expressed as means ± standard deviation of  $n = 3$  independent experiments with  $N = 180$  animals per treatment group.



polyamine levels of male and female *D. melanogaster* that were raised on control and experimental diets following the three feeding protocols E, A, and L by HPLC analysis. As shown in Figure 7A–F, neither curcumin nor BPA supplementation affected the polyamine pattern (i.e., the putrescine, spermidine, and spermine levels) of fruit flies irrespective of the applied feeding protocol.

### 3.9 | Curcumin supplementation led to enhanced polyamine levels in spleen, but not in other murine tissues

In a previous study, we found that curcumin supplementation led to a reduced iron status in mice.<sup>24</sup> Due to the mentioned reports on the relationship between iron, curcumin, and polyamines in mammalian cells,<sup>27–29</sup> we used various tissue samples of these mice to examine whether dietary curcumin and the concurrent reduced iron content had an impact on the polyamine content/metabolism. As shown in Figure 8A, the polyamine patterns of curcumin supplemented mice were not changed in liver and colon. Most notably, the spermidine level in spleen was significantly increased from  $1.15 \pm 0.16$  to  $1.53 \pm 0.18$  nmol/mg. These polyamine levels are in good accordance with the corresponding transcript levels of polyamine metabolism key enzymes in colon, liver, and spleen (Figure 8B), the latter tissue exhibiting significantly increased *odc* and *smo* expression.

## 4 | DISCUSSION

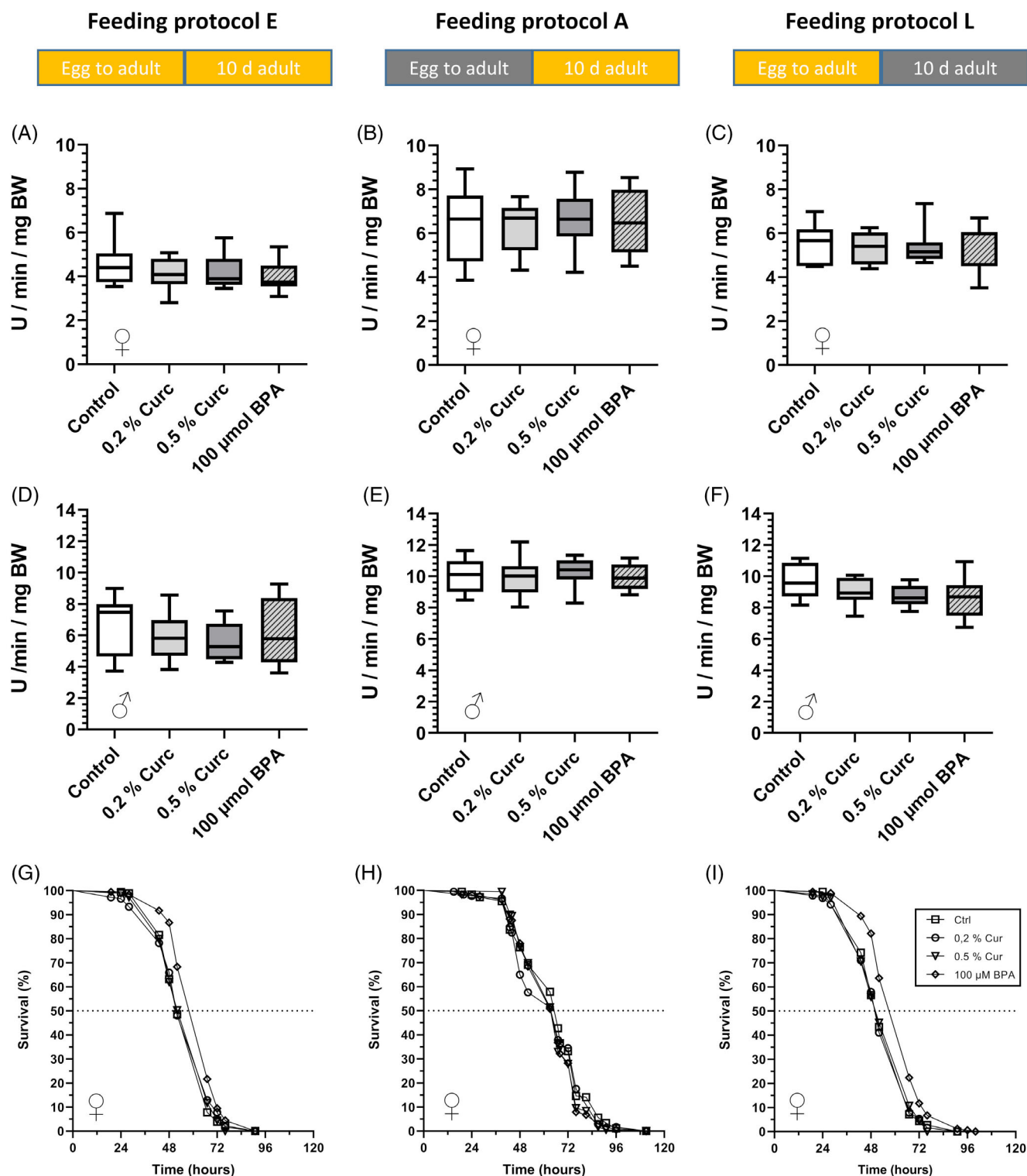
In the present study, we demonstrate that curcumin supplementation significantly reduced the iron and cobalt levels in adult *D. melanogaster* and to a lesser extent also affected their manganese and zinc content. The metal chelating properties of curcuminoids are well known and iron deficiency in response to curcumin feeding has been previously demonstrated for rodents.<sup>23–25</sup> In addition, a recent clinical case report found strong evidence for an iron deficiency anemia caused by high-dose turmeric supplementation.<sup>46</sup> The in vitro stoichiometry of the Fe(II)-curcumin complex in water at a pH 5.7 was found to be 1:1,<sup>47</sup> while BPA is known to form complexes with iron(II) under defined in vitro condition with a binding stoichiometry of 3:1.<sup>48</sup> The iron content of our *Drosophila* diet was determined to be  $\sim 50$   $\mu$ M. However, since the administration of 0.2% curcumin (equivalent to about 5 mM curcuminoids) had a smaller effect on the iron content of fruit flies than 100  $\mu$ M BPA, we conclude that BPA is the considerably more efficient iron chelator in vivo compared to curcumin.

Iron is an essential trace element and cofactor for heme and Fe-S-cluster proteins and, hence, involved in a variety of biochemical processes. Accordingly, studies using loss-of-function mutants of, for example, the *ferritin* genes (*Fer1HCH*, *Fer2LCH*), which function in absorption, transport, and storage of iron, have demonstrated that iron is also an indispensable micronutrient for embryogenesis of *D. melanogaster*.<sup>49</sup> Moreover, a knock-down of *Drosophila* ferritin specifically in the midgut caused a systemic iron deficiency of more than 50% that was accompanied with a delayed larval development and drastically reduced eclosion rate.<sup>50</sup> Despite of that, even the considerable reduction of the iron level by BPA treatment did not interfere with the developmental process of the fruit fly larvae in our and the abovementioned previous study.<sup>50</sup> Accordingly, the delayed larval development in the presence of curcumin is most probably related to the reduced food intake and not to the reduction of the iron content. It is well known that the larval developmental time of holometabolous insects is adapted to the current feeding conditions in order to accumulate sufficient resources for the subsequent metamorphosis.<sup>51</sup> In line with this, BW and body composition (i.e., the TAG to protein ratio) of the curcumin-fed larvae were similar to those of the controls and the pupation period during which food is not ingested was not affected by curcumin supplementation.

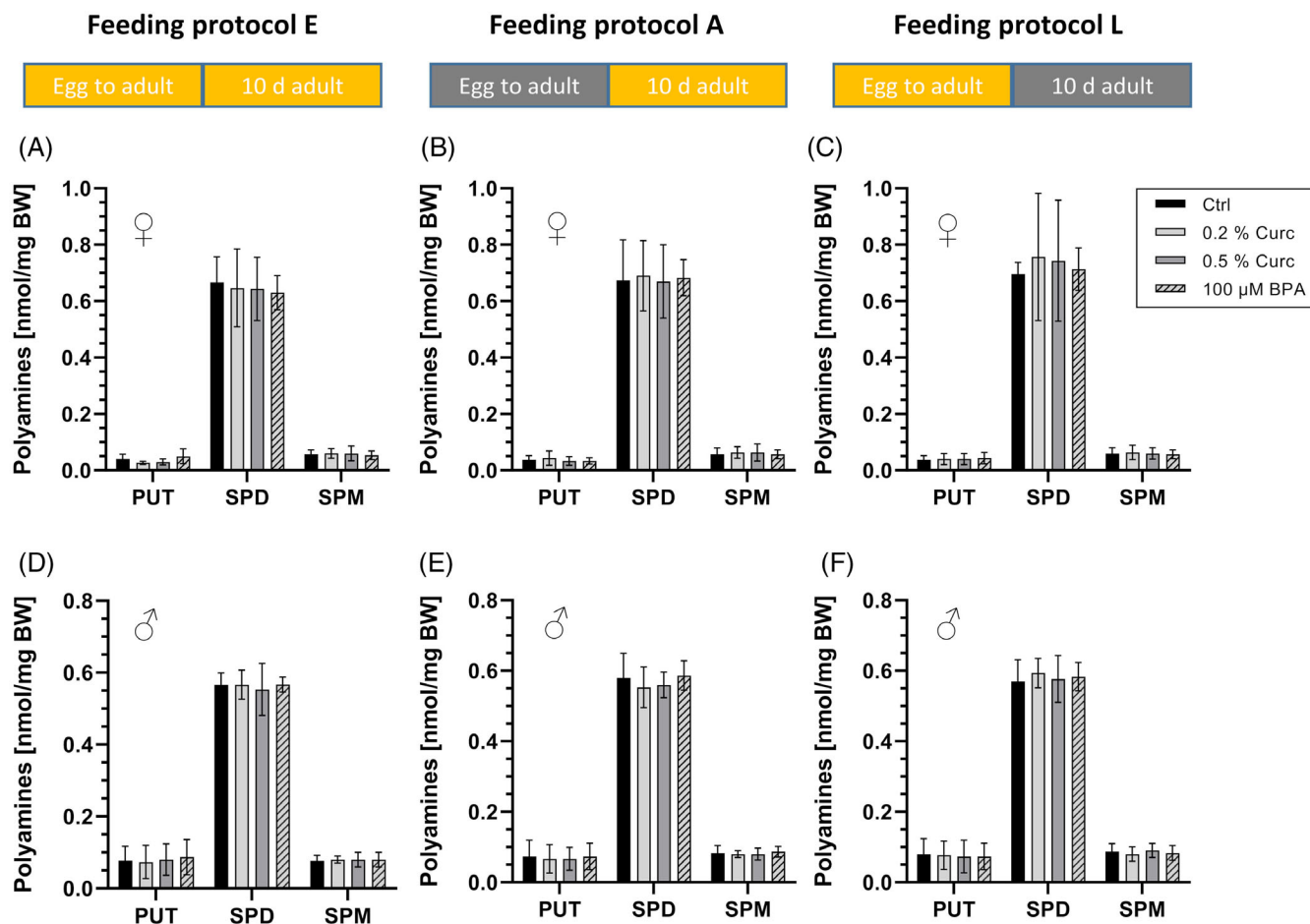
The markedly decreased iron levels in curcumin- or BPA-treated *D. melanogaster* were accompanied by a decreased level of the porphyrin/heme precursor molecules aminolevulinic acid and proporphyrinogen, although in any case this effect did not reach statistical significance. Moreover, heme degradation products were found more frequently in curcumin and BPA treated fruit flies than in the controls. Remarkably, iron deficiency and curcumin treatment, respectively, have been demonstrated to lead to an increased expression of heme-degrading heme oxygenase in mice,<sup>52</sup> cell culture,<sup>53</sup> and *D. melanogaster*.<sup>54</sup> Nevertheless, even a residual iron content of only 30% after BPA treatment (when compared to control flies) was still sufficient to maintain the catalase activity level determined for adult control flies. Since heme-dependent catalase plays a pivotal role in the detoxification of  $H_2O_2$ ,<sup>39</sup> it is reasonable that the iron-depleted flies did not exhibit a reduced tolerance toward this oxidative stressor. The even enhanced tolerance toward  $H_2O_2$  in BPA-treated females with significantly lowered iron content may be related to the Fenton reaction, in which Fe(II) as a catalyst converts the relatively modest  $H_2O_2$  into the highly reactive cell-damaging hydroxyl radical.<sup>55</sup>

The exposure to curcumin or BPA during larval development was required and, remarkably, also sufficient to





**FIGURE 6** Curcumin and bathophenanthroline treatment did not reduce catalase activity and tolerance toward  $\text{H}_2\text{O}_2$  in *D. melanogaster*. Fruit flies were reared according to the E-, A-, and L-feeding protocols, respectively (yellow bars indicate periods on experimental curcumin- or BPA-supplemented diets, gray bars periods on control diet). After eclosion and 3 days of mating, the adult animals were separated by sex. (A–F) For the determination of the catalase activity, flies were harvested on day 10 and lysed. The supernatant was used in the catalase assay. Data are presented as boxplots with whiskers indicating minimal and maximal values from three independent experiments performed in triplicate with  $N = 90$  animals per condition. (G–I) Stress tolerance assays were carried with 10 day old adult females. To this end, flies were starved for 2 h, before they were transferred to new vials containing a filter soaked with either a 5% sucrose solution only or a 5% sucrose solution with 10%  $\text{H}_2\text{O}_2$ . The survival of the animals was monitored over time. (Mantel-Cox Log-rank test). Data represent the means of three independent experiments performed in triplicate with  $N = 180$  animals per conditions.



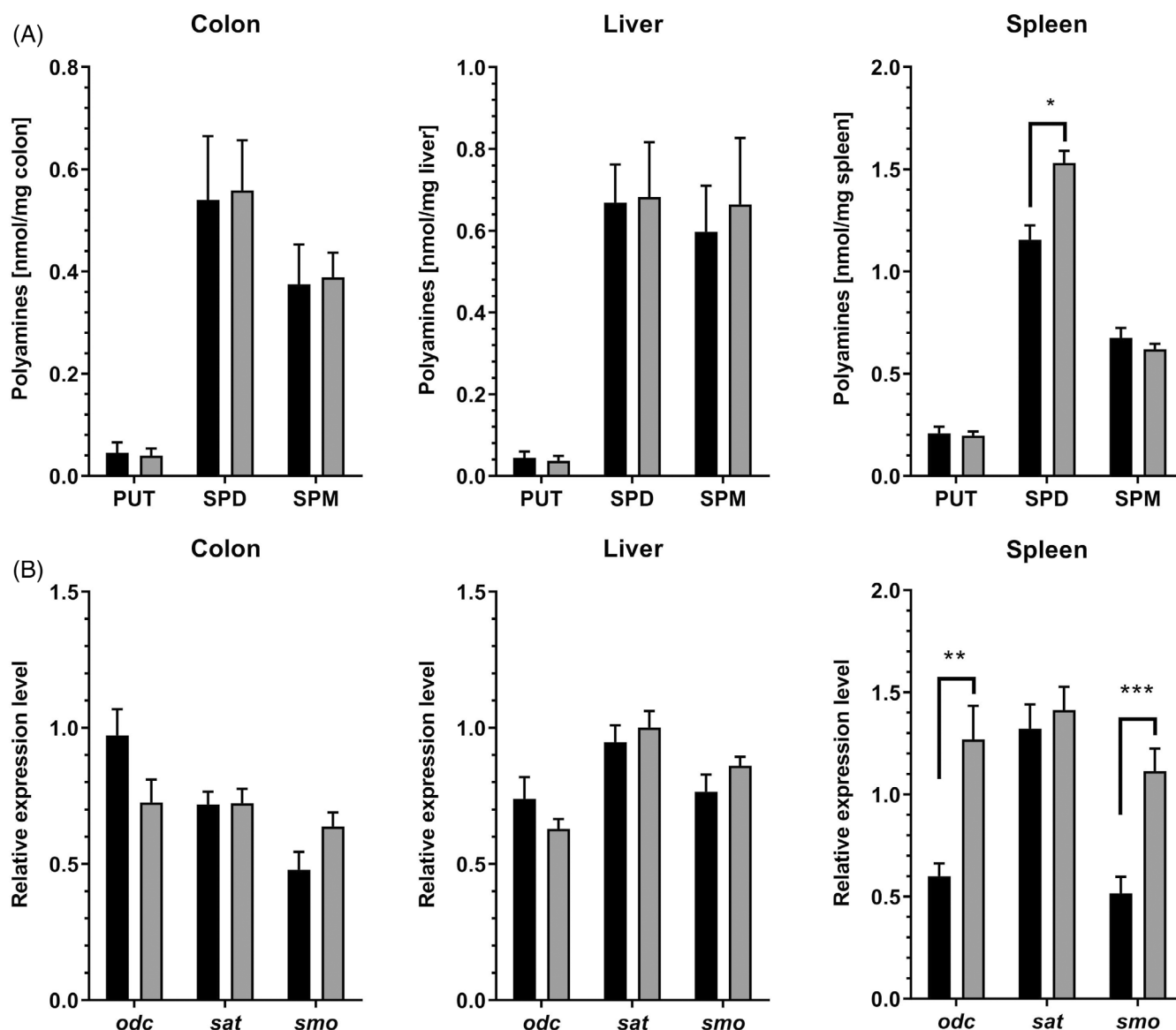
**FIGURE 7** Polyamine levels of *D. melanogaster* were not affected by curcumin or a bathophenanthroline treatment. Fruit flies were reared according to the E-, A-, and L-feeding protocols, respectively (yellow bars indicate periods on experimental curcumin- or BPA-supplemented diets, gray bars periods on control diet). After eclosion and 3 days of mating, the adult animals were separated by sex. On day 10, the animals were harvested to determine the polyamine levels by HPLC analysis. Data represent the means  $\pm$  standard deviation of three independent experiments performed in duplicate with  $N =$  animals. Two-way ANOVA (Kruskal-Wallis test) with Dunnett multiple comparisons correction. PUT, putrescine; SPD, spermidine; SPM, spermine.

induce the observed drop in the iron content of adult fruit flies, and this drop could not be reversed in the adult flies during 10 days of feeding on control diet. Accordingly, these data suggest that *D. melanogaster* incorporates its iron storage mainly during the larval stage. Consistently, the iron levels of adult fruit flies were elevated after they had been exposed to increasing concentrations of dietary iron exclusively during their development period.<sup>56</sup> The composition of early life diet has been frequently reported to affect life-history traits in adult fruit flies.<sup>57,58</sup> In this regard, it is of interest that larval curcumin supplementation was previously reported to be sufficient to extend the lifespan of adult *D. melanogaster*.<sup>6</sup> Hence, it can be speculated that the ability at least of young adult *D. melanogaster* is limited to substantially modulate the iron content, which has been already set during their larval stages. This is in line with previous reports showing that the iron absorption

and transport functions of ferritins are particularly important during embryonic development.<sup>50,59</sup> However, Massie et al.<sup>60</sup> demonstrated that iron accumulates in older adult *Drosophila* during the aging process.

Iron and zinc exhibit a physiological relevant interaction in mammals, influencing each other in terms of absorption and tissue accumulation.<sup>61</sup> In line with this, zinc levels were elevated in curcumin-treated fruit flies with reduced iron contents. However, this was not the case in BPA group, although their iron contents were even lower than in the curcumin-fed fruit flies.

Both, curcumin treatment and a reduced iron content were reported to change the polyamine pattern in mammalian cell cultures.<sup>27–29</sup> However, curcumin supplementation and the accompanied lowered iron levels did not affect the polyamine levels in the fruit fly. This is in line with the data obtained here for murine liver and colon tissues derived from a former curcumin feeding study,<sup>24</sup>



**FIGURE 8** Curcumin treatment altered the polyamine pattern and the expression of genes involved in spermidine production in murine spleen tissue. Tissues derived from a former 0.2% curcumin supplementation mice study<sup>24</sup> were analyzed for (A) their polyamine content and (B) their transcript levels of related key genes. C57BL/6J mice were sacrificed after a 28 week supplementation period. PUT, putrescine; SPD, spermidine; SPM, spermine; odc, ornithine decarboxylase; sat, spermidine/spermine *N*-acetyltransferase; SMO, spermine oxidase. Bars represent the means  $\pm$  standard deviation with  $n = 6$ –14 animals for polyamine determination and  $n = 10$ –14 animals for qPCR performed in duplicate (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , *t*-test).

however, with the exception of the spleen which exhibited significantly elevated spermidine levels. The concurrent upregulated *odc* and *smo* transcript levels in spleen tissue indicate an induced spermidine synthesis via ornithine conversion into putrescine and by mobilization of spermidine from spermine by direct oxidation via SMO, respectively. Since cell proliferation is associated with an increased cellular spermidine concentration, these data are in accordance with the increased spleen mass of the curcumin-treated mice.<sup>24</sup> In mammals, the majority of the organismic iron pool is required for erythropoiesis.

Being a hematopoietic organ during early embryogenesis, the spleen usually loses this function after birth and becomes an important immune organ. However, recent studies suggest that under certain pathological conditions including iron-deficiency anemia the spleen can be stimulated to restore hematopoietic function. Accordingly, in cases of compensatory hematopoiesis outside the bone marrow, an enlarged splenic mass was frequently observed.<sup>62,63</sup>

Our supplementation studies revealed that curcumin and BPA treatment also caused a notable decline of the

cobalt content in fruit flies. Like iron, cobalt is a first-row transition metal so both share several physico-chemical properties. Consistently, the two metals were shown to competitively inhibit each other in intestinal absorption from food.<sup>64</sup> Similar to the results obtained for iron, we found that curcumin exposure during larval development was required and sufficient to induce the decline in cobalt content of adult fruit flies, and could not be restored by the feeding on control diet during 10 days adulthood. In contrast, fruit flies that were BPA-treated during larval development were able to replenish and even increase their cobalt levels when the eclosed adult flies were transferred to control diet. Why the effects of curcumin and BPA on the cobalt content differ here is unclear and needs further investigations. It is highly likely that the metal-chelating property of curcumin and also of BPA is responsible for the observed in vivo effects on the cobalt status of fruit flies. Through this mechanism, curcumin supplementation may mitigate cobalt toxicity<sup>65,66</sup> and possibly inhibits the recently reported cobalt-dependent epithelial-mesenchymal transition in tissue remodeling.<sup>67</sup>

Cobalt is the active center of the cobalamin vitamin B<sub>12</sub>, and, hence, an essential ultra-trace element in animals. Gut microbiota represents a relevant source of *Drosophila's* vitamin B<sub>12</sub> supply.<sup>68</sup> Therefore, it can be suggested that curcumin and BPA treatment most probably also interferes with the cobalamin synthesis of intestinal bacteria, which in turn may affect the vitamin B<sub>12</sub> status of the fruit fly. The enzyme activities of methionine synthesis and methylmalonyl CoA mutase depend on cobalamin coenzymes, which is why vitamin B<sub>12</sub> deficiency is accompanied with increased homocysteine and methylmalonic acid levels.<sup>69</sup> However, our metabolomics analyses did not reveal any alterations in the methylmalonic acid level in curcumin- or BPA-treated fruit flies. The role of cobalt and vitamin B<sub>12</sub> in *D. melanogaster* has not been well studied and it is remarkable that established holidic *Drosophila* diets do not contain cobalt or cobalamines.<sup>70</sup>

Our MS data suggest that curcumin was efficiently taken up by female fruit flies, because curcuminoids and their derivatives and degradation products were detectable. The appearance or elevation of curcuminoids and their derivatives and degradation products indicate that the supplemented curcuminoids are further metabolized by the female fly and/or its gut microbiota. In particular, hexahydrocurcuminol (C<sub>21</sub>H<sub>28</sub>O<sub>6</sub>), ferulic acid (C<sub>10</sub>H<sub>10</sub>O<sub>4</sub>), and feruloyl methane (C<sub>11</sub>H<sub>12</sub>O) were significantly enhanced by curcumin feeding in female fruit flies. Likewise, in a human curcumin study, hexahydrocurcumin, which is mainly produced from curcumin in the liver and/or by intestinal microbiota,<sup>45</sup> as well as

ferulic acid and ferulic acid derivatives, which are formed as products in the curcumin degradation pathway,<sup>2</sup> were detected in human urine.<sup>45</sup> Similar to our data, the curcuminoids curcumin and demethoxycurcumin have been identified in L3 larvae of *D. melanogaster* in a previous curcumin feeding study.<sup>44</sup> Remarkably, the main proportion (about 2/3) was associated with the gut compartment, which points to a limited bioavailability of curcumin in fruit flies larvae. This is in line with studies in mammals, where curcumin has been shown to exhibit a low bioavailability and to undergo rapid molecular transformation, for example, by auto-oxidation or cellular detoxification pathways.<sup>12</sup> Against the background of the low uptake rates, we assume that the influence of curcumin feeding on the trace metal content of fruit flies detected in both sexes is mostly attributable to the chelating activity of curcuminoids within the digestive tract.

## 5 | CONCLUSION

Curcumin possesses numerous health-promoting properties, which is why it is considered a promising natural substance with broad therapeutic and nutraceutical fields of application. However, the low stability and poor bioavailability often limit the effect of curcumin in vivo. On the other hand, there is increasing evidence that high dosing and improvement of pharmacokinetic and pharmacodynamic properties may lead to unwanted side effects such as hepatotoxicity and iron deficiency. Our study with the model organism *D. melanogaster* revealed that dietary curcuminoids are absorbed and, similar to the situation in mammals, further metabolized by fruit flies. Furthermore, our data confirmed the in vivo iron-chelating effect of curcumin and unveil that the status of other trace elements including cobalt, zinc and manganese is also affected. Accordingly, future curcumin supplementation studies should consider the impact on these and possibly other trace elements that may be associated with negative physiological consequences, especially under conditions of poor micronutrient supply or high micronutrient demand. It is quite remarkable that the partly strong changes in the status of the investigated trace elements were found to be not responsible for the curcumin-induced retarded development of the fruit fly and did not alter the stress tolerance of adult flies toward H<sub>2</sub>O<sub>2</sub>. In mammals with a drastically higher iron requirement than *Drosophila*, the dose of 0.2% curcumin was previously shown to be sufficient to elicit iron-deficiency and an enlargement of the spleen.<sup>24</sup> The associated elevated spermidine production found here, is most probably an organ-specific indirect response due to an increased cell proliferation. Since no impact on the



polyamine profile was determined in either the fruit fly or the other mouse organs studied, we suggest that the polyamine metabolism is not a direct curcumin target in vivo.

## AUTHOR CONTRIBUTIONS

Tobias Jöckel conducted the experimental fruit fly work. Dawn Chin carried out the mouse study. Tobias Demetrowitsch performed the mass spectrometry. Gerald Rimbach, Karin Schwarz, and Kai Lüersen did the supervision. Tobias Jöckel, Tobias Demetrowitsch, and Kai Lüersen did data curation. Gerald Rimbach and Kai Lüersen designed the study. Kai Lüersen wrote the text. Tobias Jöckel, Tobias Demetrowitsch, and Gerald Rimbach revised the text.

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## CONFLICT OF INTEREST STATEMENT

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

## DATA AVAILABILITY STATEMENT

Additional data that supports the findings of this study are available in the supplementary material of this article. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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