To study whether offspring sex is related to the amount of resources invested in eggs we performed an experiment on zebra finches, *Taeniopygia guttata*. By manipulating their food supply, we forced two groups of females either to increase or to decrease investment in subsequent eggs. Since zebra finches are sexually dimorphic and the reproductive value of the sexes may vary with maternal nutritional status, we predicted that females would adjust the sex of their offspring to egg quality. Females that received poor-quality food for 7 weeks before egg laying, then food supplemented with proteins after they laid the first egg, significantly increased the mass of subsequent eggs. An increase of egg mass with laying sequence was less pronounced in females that received high-quality food before laying and experienced food deterioration after starting to lay. The proportion of sons in subsequent eggs tended to increase in the latter group (although this was marginally significant) but was not related to laying sequence in the other group: these patterns differed significantly between the groups. Offspring sex was not related to egg mass, but newly hatched male chicks were heavier than female chicks. Furthermore, the hatching success of male eggs was lower than that of female eggs. We suggest that differential hatching success of the sexes and sex differences in mass at hatching may constitute important factors shaping brood sex ratios.
dimorphism in egg size has also been reported in mountain white-crowned sparrows, *Zonotrichia leucophrys ornitha* (Mead et al. 1987), the house sparrow, *Passer domesticus* (Cordero et al. 2000), and the mallard, *Anas platyrhynchos* (Cunningham & Russell 2001). In all these species, bigger eggs contained male embryos. However, the only two experimental studies of this question, on lesser black-backed gulls, *Larus fuscus* (Nager et al. 1999) and on great skuas, *Catharacta skua* (Kalmbach et al. 2001), failed to show a difference in egg size between the sexes.

We performed an experiment to investigate whether offspring sex is related to the amount of resources invested in eggs in laboratory zebra finches. To differentiate female investment in eggs, we kept two groups of females either on low- or high-quality food for 7 weeks. Then we mated them and, after the female laid the first egg, we reversed the food regime. Our goal was to differentiate the quality of eggs within broods and between experimental groups, then to evaluate whether females were able to adjust the sex of their offspring to egg quality. We predicted that the quality of food available to females before egg laying should affect the size of their first eggs, and that reversing food quality should evoke size changes of subsequent eggs. Specifically, we predicted an increase in egg size with laying order in females whose food condition had improved and a decrease in egg size in females whose food condition had deteriorated. Thus, if female zebra finch chicks are more sensitive to malnutrition (Kilner 1998), and the female is able to determine the egg’s sex at the time of egg formation, we predicted that daughters should be produced in large eggs. We also predicted that changes in egg size with laying order should accompany changes in the proportion of sons, which should increase with laying order in the group of females in which food quality deteriorated and decrease in the group of females experiencing food improvement.

**METHODS**

We obtained zebra finches from local breeders. Birds were kept in an air-conditioned room, under a 13:11 h incandescent light:dark photoperiod at 21 ± 2°C. Before the experiment, all birds were maintained in an aviary, where they could mate freely and rear at least one brood. Then we separated the sexes and randomly assigned 24 females to two equally large groups with different food regimes. The first group was fed ad libitum with a standard mixture of seeds (Minifit, Perbo), that contained ca. 12% protein. The second group received the same seed mixture along with a mixture of hard-boiled egg chopped with biscuits and finely grated carrot (2 g per female every morning). The protein content of this mixture was ca. 44%. Birds also received a cuttlebone and grit. Vitamins C, A, B1, B6, B12, D3, K (Multivit, Tropical) were added to the water every second day.

Seven weeks after we established the experimental groups, we placed females into visually separated, individual cages (75 × 30 cm and 40 cm high) equipped with external nestboxes, paired them with males and provided them with nest-building material. After the females were mated, we inspected the nestboxes every morning. All new eggs were numbered and weighed with an electronic balance (0.01 g).

Food regimes were maintained until the first egg was laid. On that day we reversed the food regime: the females that had been fed only seeds received the protein-rich mixture, while the females previously fed the protein-rich diet received only seeds. Thus, after the first egg was laid, food quality improved in one group of females and deteriorated in the other group.

Knowing the date that incubation began, we calculated the expected hatching date. From that day until all eggs hatched, we inspected nests every hour to determine which hatchling came from which egg. During nights (between 2000 and 0800 hours), eggs were transferred to an incubator chamber (humidity about 70%, temperature 36.4°C). Most eggs hatched between 0800 and 1100 hours and only a few before 0800 hours. Newly hatched chicks were immediately weighed, marked by cutting one of their nails and returned to the nest. At the age of 2 weeks, nestlings were ringed with individually numbered aluminium rings.

We determined the sex of the fledglings by plumage characteristics (N=48). The sex of embryos that failed to hatch, and of nestlings that died before we could assess sex by plumage, was determined with molecular techniques (N=42; Griffiths et al. 1998). We determined the sex in 90 of 99 eggs. One egg dried out because it was apparently unfertilized, or the embryo died at an early stage of development and its DNA degraded.

We extracted DNA from brain tissue or blood, using NucleoSpin C+T kit (Macherey–Nagel) following the manufacturer’s protocol. To amplify the CHD-W and CHD-Z genes from sex chromosomes, we used the polymerase chain reaction (PCR) with primers P2 and P8 (Griffiths et al. 1998). The temperature profile in PCR was as follows: hotstart at 94°C; initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, template extension at 72°C for 1 min, and final extension at 72°C for 5 min. The reagents were in the following concentrations: MgCl₂ 1.5 mM; dNTP 0.2 mM each; polymerase 0.5 U/μl and PCR buffer (Fermentas) in total volume of 10 μl. PCR products were separated by electrophoresis at 5 V/cm for 1.5 h on 3% agarose gel stained with ethidium bromide. We visualized them by UV transillumination and sexed birds according to the presence of two bands (350 and 384 bp) for a female and one band (350 bp) for a male. The person scoring the gel was blind to the identity of the samples.

Two females in each group deserted their clutches after laying the first egg. We excluded them from most of the analyses. One female deserted her clutch just before hatching. In two clutches we observed laying gaps. Since we could not be sure whether these females laid no egg that day, we numbered the subsequent eggs after the gap as though the lacking egg had been laid.

To assess the sources of variation in egg mass and hatchling body weight, we analysed the data with General Linear Model. We introduced female identity to account for variation attributed to female characteristics,
and nested this factor in the experimental group because a particular female belonged to only one of the two groups. Because we predicted that the experimental procedure would cause changes in egg mass and hatchling weight with laying sequence, we introduced egg number as a covariate.

Logistic regression with a logit link function was used to model the probability that an egg contained a male or female embryo and the probability of successful hatching as binary response variables (0 or 1; female or male and unhatched or hatched, respectively). The egg number, experimental group and female identity nested in the experimental group were independent variables. Egg sex was also introduced as an independent variable in the analyses of hatching success. In the latter analyses, non-significant interactions were removed from the model. Because the logistic regression is sensitive to single binary values occurring in some of the categories, we excluded from the analysis the sixth eggs in the group experiencing food deterioration and seventh eggs in the other group. In total we excluded four eggs. Clutches of six and seven eggs were underrepresented (Fig. 1b, c), which caused large differences in variance that heavily affected the outcome of the statistical tests.

Work was carried out under licence from the Local Ethical Committee at the Jagiellonian University.

RESULTS

During the 7 weeks after we established the experimental groups, females from both groups gained weight on their original diets. Protein-supplemented females gained slightly more weight than did nonsupplemented females (0.9 g versus 0.2 g, respectively), but the difference in weight gain between groups was not significant (ANOVA with repeated measures: $F_{1,32}=1.15$, $P=0.3$).

As predicted, differences in food quality available to females from the two experimental groups resulted in differences in the number and size of their eggs. Females fed the protein-poor diet before egg laying laid smaller clutches (4.5 versus 5.4 eggs on average; ANOVA: $F_{1,18}=4.89$, $P=0.04$), consisting of lighter eggs (1.074 versus 1.167 g, on average; Table 1, Fig. 1a), than females fed high-quality food. Subsequent eggs in the laying order became heavier in both groups, but there was a significant interaction between experimental treatment and egg number (Table 1). The relation was much stronger in females experiencing food improvement after they laid their first egg (ANCOVA, controlling for female effect: $\beta=0.77$, $F_{1,34}=50.09$; $P<0.0001$ versus $\beta=0.43$, $F_{1,43}=9.86$, $P=0.003$; Fig. 1a).

We found no evidence that male and female eggs differed in mass (1.135 versus 1.127 g; Table 1). However, if offspring sex is related to the amount of resources that a female invests in her eggs, we would expect the sex of subsequent eggs in the laying order to follow the observed changes in egg mass. In females in which food quality deteriorated after laying the first egg, the proportion of sons tended to increase, although not significantly, with egg number (logistic regression accounting for female identity; $\beta=0.56$, $\chi^2=3.68$, $P=0.055$), but it did not increase in the protein-enhanced group (logistic regression accounting for female identity: $\beta=0.06$, $\chi^2=0.25$, $P=0.6$; Fig. 1c). The interaction between experimental group and egg number was significant, which indicates that females in these two groups responded differently to changes in food quality (Table 2). The experimental groups did not differ significantly in sex ratio. The proportion of males was 0.63 in the group in which food quality deteriorated and 0.54 in the protein-enhanced group.

Although there was no sexual dimorphism in egg size, the mass of newly hatched chicks differed significantly between male and female chicks (Table 1). Male hatchlings were heavier than female hatchlings, especially in the group in which food quality had improved after the first egg was laid (sons: 0.788 g; daughters: 0.766 g; Fig. 1b; see also the interaction between egg number and egg sex in Table 1). Furthermore, hatching success was sex-related (Table 2). Almost all females (97%) and only 82% of the males hatched, but overall hatching success did not differ between groups (85% in the group experiencing food deterioration; 78% in the protein-enhanced group).

DISCUSSION

Manipulation of food quality before and during the egg-laying period in zebra finches successfully influenced patterns of female investment in the subsequent eggs within clutches. Egg mass gradually increased with the laying order in both groups, but it was significantly more pronounced in females originally fed a poor diet that was subsequently enriched after they laid their first egg. In this group, the fifth and sixth eggs were over 20% heavier than the first egg (Fig. 1a). Despite the deterioration in food quality during egg laying, females in that group laid larger clutches and heavier eggs than did females that had been food depleted before laying. Larger clutches of these females suggest that the clutch size was predetermined at the beginning of laying, before the change in food quality was imposed. This may be as expected, since egg development takes about 4 days in zebra finches (Christians & Williams 2001). Thus, at the time of laying the first egg, the second egg was already fully developed and the third’s yolk content was developed to a large extent. Hence, the effect of deterioration of food quality would be expected to be apparent only after the third egg was laid. If an increase in egg size with laying order is a common trend in zebra finches (Williams 2001; our unpublished data; but see Zann 1996 for results in wild zebra finches), our results indeed suggest that this trend was disrupted after the third egg laid by protein-depleted females (Fig. 1a, b).

Female zebra finches may be more vulnerable to nutritional stress than males (Zann 1996; de Kogel 1997; Kilner 1998). If so, then differential investment in male and female eggs should be adaptive. Such differences in egg size between sexes have been reported in studies of different species (Mead et al. 1987; Anderson et al. 1997; Cordero et al. 2000, 2001; Cunningham & Russell 2001). We expected zebra finch females to produce sons in small
eggs and daughters in large eggs. This prediction was not supported by our results, because the sex of the embryo was not related to egg mass. However, we found that the mass of newly hatched chicks differed between the sexes (Table 1). Contrary to the above prediction, male chicks were heavier than female chicks at hatching, and this difference was more pronounced in the group in which food quality had improved after the first egg was laid (Fig. 1b). Thus, female zebra finches may be the more vulnerable sex in the face of a food shortage because they are smaller at hatching and can potentially be out-competed by their bigger brothers (cf. Oddie 2000). However, our results do not help to distinguish whether females are more sensitive because of nutritional stress or because of lower competitive abilities. The lack of sex differences in egg mass, although there was a significant effect of sex on hatchling mass, calls for an explanation. Egg mass may not necessarily reflect a female’s actual investment, if eggs differ in water and nutrient content (Williams 1994). Thus, sexual dimorphism in egg mass

**Figure 1.** The effects (X±SE) of feeding treatment and laying order on (a) egg mass, (b) body weights of male and female hatchlings and (c) proportion of males (numbers denote sample sizes; lines are the logit function fitted to data of offspring sex). Data are shown for females experiencing deterioration in food quality after laying the first egg and for females for which food quality improved.
may not be well pronounced, but the mass of newly hatched chicks may better reflect a female’s actual resource investment in eggs, providing that the sexes do not differ in the rate of embryonic development. If this is the case, our data suggest that females allocated resources differentially in sons’ and daughters’ eggs, but it was pronounced enough to be noticeable only in females kept on poor-quality food before laying.

The observed differences between the sexes in the body weights of hatchlings, although they were not observed in egg mass, could also arise because of differential hatching success between the sexes. Almost all females hatched, but male hatching success was lower (Table 2). Differential hatching success between the sexes has also been recently reported in tree swallows, Tachycineta bicolor, in which, as in our study, male embryos were more likely to die (Whittingham & Dunn 2001). We suggest that differential hatching success between the sexes, and sex-specific body weight at hatching, may constitute important factors shaping brood sex ratio.

Because of different food regimes, we predicted that females from one group would provide subsequent eggs with more resources and the others would provide fewer resources in subsequent eggs. Thus, following our predictions, offspring sex should change with laying sequence according to changes in egg quality. As expected, females receiving the protein-depleted diet after laying the first egg tended to produce more sons in subsequent eggs, but this tendency was not observed in females receiving the protein-enriched diet (Fig. 1c). If females can change the production of one sex over the other in subsequent eggs in response to immediate changes in food quality, this finding suggests that embryo sex is adjusted to the amount of resources in the egg, and not the other way around. However, the observed bias in sex ratio in response to changes in food quality may also result from the mother’s anticipation of the conditions expected during nestling rearing. If conditions deteriorate, it may pay to produce the less vulnerable sex.

Changes in egg sex with laying order have already been reported in zebra finches (Kilner 1998): as in our subsequently protein-depleted group, regardless of food availability during reproduction, female eggs were laid earlier than male eggs. If the increase in the proportion of males in subsequent eggs were typical in zebra finches, this trend was disrupted in the group of females experiencing food improvement. Kilner (1998) suggested that females were produced early in the laying sequence because they were more sensitive than males and could better compete for resources when hatched earlier. In the light of the present results, one could suggest the alternative, that females come first, because males are more sensitive because they show lower hatching success and should come later when eggs become larger (i.e. contain more resources).

The two other experimental studies of this question also failed to show differences in egg size between the sexes (Nager et al. 1999; Kalmbach et al. 2001). In these experiments, variation in egg size was elevated by egg removal, which forced females to lay extra eggs that became smaller with laying order. In lesser black-backed gulls, the probability of an egg producing a female increased with laying sequence, but only in the group of females that were not food supplemented (Nager et al. 1999). In a similar experiment on the great skua, which has reversed sexual size dimorphism, unsupplemented

Table 1. Factors influencing the mass of subsequent eggs in the laying order and body weight of hatchlings (General Linear Model analyses)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Egg mass</th>
<th></th>
<th></th>
<th>Hatchling weight</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>df</td>
<td>P</td>
<td>F</td>
<td>df</td>
<td>P</td>
</tr>
<tr>
<td>Experimental group</td>
<td>1</td>
<td>48.34</td>
<td>1</td>
<td>&lt;0.001</td>
<td>27.68</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female (experimental group)</td>
<td>18</td>
<td>5.08</td>
<td>18</td>
<td>&lt;0.001</td>
<td>4.08</td>
<td>17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Egg number</td>
<td>1</td>
<td>66.53</td>
<td>1</td>
<td>&lt;0.001</td>
<td>23.19</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Egg sex</td>
<td>1</td>
<td>0.00</td>
<td>1</td>
<td>0.991</td>
<td>4.91</td>
<td>1</td>
<td>0.031</td>
</tr>
<tr>
<td>Experimental group×egg number</td>
<td>1</td>
<td>22.91</td>
<td>1</td>
<td>&lt;0.001</td>
<td>19.73</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Egg number×egg sex</td>
<td>1</td>
<td>3.92</td>
<td>1</td>
<td>0.053</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>67</td>
<td>52</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nonsignificant interactions (all P>0.7) were removed from the model and hence are not shown.
females overproduced the smaller sex (males) at the end of the laying sequence (Kalmbach et al. 2001). This result corroborates our findings that, although egg mass might not be related to offspring sex, females adjust the sex of subsequent eggs in the laying order to the availability of resources.

In conclusion, in this experimental study we found no relation between egg mass and sex, although changes in embryo sex with laying order differed significantly between our experimental groups in the direction we predicted. Furthermore, we found sex-related differences in body weight at hatching. Male hatchlings were heavier, especially in the group experiencing food improvement after laying the first egg, and female eggs had a higher probability of hatching. These results may indicate that females can adjust the sex of the eggs to the amount of resources invested in eggs. Both sex-specific hatching success and differences between the sexes in body weight at hatching may be important in shaping brood sex ratios.

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