ECOSCIENCE

Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds[1]

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Abstract: Growing altricial birds may experience nutritional stress in the nest due to sibling competition, food restriction, or parasites. Nutritional stress may be detected through its effect on nestling growth, although genetic and maternal effects may interfere with its expression. A more direct way of estimating nutritional stress may be through measurements of organismic stress in growing birds. Heterophil/lymphocyte ratio (H/L) have been proposed as reliable physiological indicators of the stress response. Heat-shock protein (HSP) synthesis is induced by animals in response to various stressors, making the concentration of HSPs a suitable measure of organismic stress. In a study of growing pied flycatchers (*Ficedula hypoleuca*), we assessed H/L from cell counts in blood smears and levels of two HSPs, HSP60 and HSP70, from the cell fraction of peripheral blood. H/L and the level of HSP60 were significantly positively associated in nestlings of 13 days of age, while the level of HSP70 was not related to the other two measures. Small nestlings with respect to tarsus length on day 7 had higher levels of HSP60 and higher H/L values. Brood means of nestling mass, tarsus length, and wing length shortly before fledging were negatively related to both mean HSP60 level and mean H/L ratio. Brood variances in body mass and wing length on day 13 were positively associated with mean HSP60 levels but not with mean H/L values. Up to 80% of variation in some traits was explained by stress indicators.

Keywords: immune system, life history, growth, sibling competition, environmental component, size, condition.

Résumé : Les oisillons nidicoles peuvent subir des stress nutritionnels lorsqu'ils sont au nid en raison de la compétition entre les oiseaux d'une même nichée, d'une diminution de la disponibilité de nourriture ou de parasites. Un stress nutritionnel peut être décelé par ses effets sur la croissance de l'oisillon, bien que son expression puisse être masquée par des facteurs génétiques ou maternels. Il est possible d'estimer de façon plus directe un stress nutritionnel par la mesure du stress que subissent des oiseaux en croissance. Les rapports entre les leucocytes neutrophiles et les lymphocytes (N/L) ont en effet été proposés comme indicateurs physiologiques fiables de réponse au stress. La synthèse des protéines associées aux coups de chaleur (HSP) est induite chez les animaux en réponse à différents agents de stress; leur concentration peut donc servir de mesure du stress de l'organisme. Nous avons évalué chez de jeunes gobemouches noirs (*Ficedula hypoleuca*) le rapport N/L à partir de la numération cellulaire de frottis sanguins, ainsi que les niveaux de deux protéines HSP (HSP60 et HSP70), à partir de fractions cellulaires de sang périphérique. La relation entre le rapport N/L et la concentration de HSP60 était positive et significative chez les oisillons âgés de 13 jours. La concentration de HSP70 n'était toutefois pas reliée à ces deux mesures. Chez les petits oisillons (la taille était déterminée par la longueur du tarse au 7e jour de vie), les concentrations de HSP60 et les rapports N/L étaient plus élevés que chez les plus grands oisillons. Chez les nichées, les moyennes de la masse corporelle, de la longueur du tarse et de la longueur de l'aile des oisillons peu de temps avant leur premier envol étaient associées de façon négative aux moyennes des concentrations des protéines HSP60 et des rapports N/L. On a trouvé une relation positive entre les variances de la masse corporelle et de la longueur de l'aile chez les nichées au jour 13 avec la moyenne des concentrations de HSP60, mais pas avec la moyenne des rapports N/L. Près de 80 % de la variation de certains caractères était expliquée par des indicateurs de stress.

Mots-clés : système immunitaire, cycle vital, croissance, compétition entre frères et sœurs, composante environnementale, taille, condition.

Introduction

Nestlings are exposed to variable conditions with respect to nutrition during growth, which may be reflected in their levels of physiological stress (Hoi-Leitner *et al.*, 2001). Ascertaining food restriction through morphological measurements or body mass at a certain stage of development may incorporate genetic or maternal effects, which are not directly related to nutritional condition during the growth period (Merilä, 1996). Physiological and nondestructive stress indicators may be used to determine nutri-

tional stress in growing organisms. Among the most widely used are several measures derived from samples of peripheral blood. Food restriction can have important implications for leucocyte production and circulation in domestic poultry (Maxwell, 1993). The heterophil/lymphocyte ratio (H/L) is widely accepted as a reliable and accurate physiological indicator of stress (Maxwell & Robertson, 1998). Its relevance for studies of nutritional stress in wild populations has recently been explored (Horak, Ots & Murumägi, 1998; Ots, Murumägi & Horak, 1998). Heat shock proteins (HSPs), also called stress proteins, are evolutionarily highly conserved molecules that help cells in recovering from

stress situations by correcting misconfigurations in protein structures (Morimoto, 1991). Although thermal stress was the initial focus of studies on HSP function (Coleman, Heckathorn & Hallberg, 1995; Krebs & Feder 1997), disease (Garbe, 1992), inflammation (Macario, 1995), and pollution (Delmas *et al.*, 1996) also can induce increased HSP production. Recently, HSP levels have been used to evaluate the stress inflicted by parasites (Merino *et al.*, 1998; 2001) and pollution (Eeva *et al.*, 2000) on nestling birds.

Here we have tried to compare both indicators of physiological stress in a sample of pied flycatcher (*Ficedula hypoleuca* Pallas) broods. The first issue raised is whether stress-mediated changes in leucocyte production and circulation are related to stress protein synthesis. To our knowledge, this information is not available for any species, although an immunological challenge led to increases in the concentration of stress proteins in piglets (McComb & Spurlock, 1997). A significant association between H/L and HSP concentrations would reinforce the use of both stress indicators for field studies of organismic stress.

The second issue is whether stress indicators are related to patterns of nestling growth. Merino *et al.* (1998) detected a significant association between HSP levels and wing length in nestling house martins (*Delichon urbica* L.). Nestling mass and morphology are the product of the complex interaction of genetic, maternal, and environmental effects (Merilä, 1997) and are not independent measures of nutritional stress. Environmental variation is normally increased by stress conditions in birds (Hoffmann & Merilä, 1999). If stress imposed by food restriction is important in determining growth, the genetic potential of organisms will not be reached under poor nutrition (Alatalo, Gustafsson & Lundberg, 1990; Gebhardt-Henrich & van Noordwijk, 1991) Thus, a strong association of stress indicators with nestling morphology may reflect a reduced evolutionary potential of certain traits.

A third question relates to the impact of food restriction on food allocation among siblings by parents. On top of stress directly effected by food restriction, sibling competition may induce stress through enforced begging activity or movements leading to strategic positioning in the nest (Ramos Fernández *et al.*, 2000; Brzek & Konarzewski, 2001). Increased competition may lead to a more unequal food distribution among siblings, thereby increasing variance in growth patterns (Nuñez de la Mora, Drummond & Wingfield, 1996). The association of increased stress with increased variance in nestling traits may reveal the importance of sibling competition in determining intra-brood growth patterns.

Methods

The study was conducted in 1999 in a deciduous forest of Pyrennean oak (*Quercus pyrenaica* Willd.) at 1,200 m a.s.l. in the vicinity of La Granja, Segovia province, central Spain (40° 48' N, 4° 01' W). A study of a population breeding in nestboxes in this area has been conducted since 1991 (Sanz & Moreno, 2000). Nestboxes (125 mm × 117 mm bottom area) are cleaned every year after the breeding season. Every year, the nestboxes are checked for occupation by pied flycatchers, and the dates of clutch initiation, clutch sizes, and number of fledged young are determined.

The pied flycatcher is a small (12-13 g) hole-nesting passerine of European woodlands. It is a summer visitor that adapts readily to breeding in artificial nestboxes. Egg laying in the population being studied typically begins in late May, and clutch sizes range from 2 to 8 eggs, with a mode of 6 eggs (mean 5.73). The female incubates alone and receives part of her food from her mate. Young are brooded by the female only up to the age of 7 days (Sanz & Moreno, 1995). Both sexes feed the young (Moreno *et al.*, 1995). Young fledge within 14-16 days of hatching. This occurs in the second half of June in our study area. There are no second clutches in our study population.

A sample of 22 nests were used for the study of nutritional stress. On day 7 (hatching day = day 0), the tarsus length of chicks (defined as distance between joints) was measured with a digital caliper to the nearest 0.01 mm (except in 4 broods), and their mass was obtained with a Pesola spring balance (0.1 g). All chicks were ringed with numbered aluminium bands (DGCN bands, ringing permit by regional authorities). On day 13, all surviving chicks were again measured and weighed in the same manner. Wing length was measured with a stopped ruler to the nearest mm (Svensson, 1984). Blood was sampled from the brachial vein into heparinized capillary tubes to use for smears and for centrifugation for HSP analyses.

Leucocytes form the basis of the immune system, and their main function is protection against foreign pathogens. Lymphocytes and heterophils are the most abundant types of leucocytes in avian blood (Campbell, 1995). Heterophils are bactericidal phagocytising cells that enter tissues during the inflammatory response (Maxwell & Robertson, 1998). They are non-specific immune cells, in contrast to the highly specific response of lymphocytes (Jurd, 1994). Davison, Rowell and Rea (1983) and Gross and Siegel (1983) described a ratio calculated from the proportions of heterophils and lymphocytes present in the circulation of domestic fowl as a measure of stress. The H/L ratio has now become widely accepted as a reliable and accurate physiological indicator of the stress response (Maxwell & Robertson, 1998). To estimate H/L, a blood smear was obtained from the brachial vein of chicks. A drop of blood was smeared on individually marked microscope slides, airdried, fixed in absolute ethanol, and stained with Giemsa. Slides were examined under microscope to estimate the proportions of different types of leucocytes. Examination was arrested when 100 leucocytes, excluding thrombocytes, had been found under 1,000× magnification with oil immersion (thrombocytes normally present an irregular, aggregated distribution). Fields with similar densities of erithrocytes were scanned for all individuals. H/L was estimated from the numbers of heterophils and lymphocytes per 100 leucocytes obtained in these counts. Granulocytes were doublechecked for identification using 1,000× magnification. The heterophil/lymphocyte (H/L) ratio has been used as an index of stress (Gross & Siegel, 1983; Maxwell, 1993) and has shown a low measurement error in field situations (Ots, Murumägi & Horak, 1998). Given the presence of trypanosomes in peripheral blood of pied flycatcher nestlings (Merino, Potti & Moreno, 1996) and the effect of hematozoa on HSP levels (Merino *et al.*, 2002), blood smears were screened for trypanosomes with a 200× objective. To con-

trol for possible nonrandom distribution of parasites, half of each smear was entirely scanned. Thus, in most cases more than 300 fields were scanned (about 15 minutes smear[-1]). No intraerthrocytic hematozoa were detected in this or previous studies of pied flycatcher nestlings (Merino & Potti, 1995). We considered broods to be infected by trypanosomes when at least one nestling was infected (see Merino, Potti & Moreno, 1996 for rationale).

Capillaries were centrifuged for 10 minutes at 12,000 r.p.m. less than 2 hours after collection in the field. After centrifugation, cellular and plasma components were separated and immediately frozen. We analyzed the cellular part of blood in search of heat shock proteins by means of a Western blot. This technique is usually and currently employed for detection and quantification of HSPs (Hotchkiss *et al.*, 1993; Flanagan *et al.*, 1995; Knowlton *et al.*, 1998; Merino *et al.*, 1998; Martínez *et al.*, 1999). Blood cells were homogenized in 0.2 mL distilled water by a sonicator. This homogenate was centrifuged at 14,000 r.p.m. for 20 minutes at 4°C. The supernatant was collected, and the protein concentration determined (Bradford, Bio-Rad). Samples (100 µg well[-1] for HSP70 and 150 µg well[-1] for HSP60) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis and electroblotting were done according to Laemli (1970) and Towbin, Staehelin, and Gordon (1979), respectively. Blots were tested with antisera using as primary monoclonal antibodies anti-HSP70 (1/5,000, clone BRM 22, Sigma) and anti-HSP60 (1/1,000, clone LK1, Sigma). The use of these antibodies in the studied species is validated because two immunoreactive bands with the appropriate molecular weights of around 60 and 70 kDa were obtained by using western blot. The peroxidase-conjugated secondary antibody (Anti-mouse specific for Fc region, Sigma) was used at 1/6,000 dilution. Densitometric quantification of immunoreactivity was obtained with an image analyser (Quantity One, Biorad). HSP levels were expressed as arbitrary units of optical density per area (O.D. area[-1]). Prior to this study, experiments were carried out to demonstrate linearity of immunoblot results at the conditions to be used. Evaluation of test results on pied flycatcher blood samples by regression analysis revealed in all cases a high correlation ($r > 0.95$) between observed immuno-reactivity and amount of protein applied in the test. In order to minimise differences between blots, all samples were processed and analyzed at the same time. Previous experiments showed low variation in the value obtained for the same samples being run on different gels by using this method ($F_{9, 12} = 1.11$, $p = 0.44$; J. Martínez, unpubl. data).

When relating stress to growth, brood means were analyzed. These tests are accordingly conservative. H/L ratios have been logarithmically transformed to obtain normal distributions.

Results

Mean H/L of nestlings was 0.45 ± 0.49 ($n = 78$ nestlings, range 0.05-3.0). This level is very similar to that of adults in this population (Moreno *et al.*, 2002). Levels of HSP60 and H/L ratios of individual nestlings were signifi-

cantly positively correlated (Figure 1). The same was true for mean brood values ($r_{20} = 0.66$, $p = 0.002$). The level of HSP70 was not related to HSP60 level or to H/L values ($p > 0.10$). Trypanosomes were detected in only three nestlings of three different broods. There were no significant differences between the three infected broods and the rest with respect to levels of either HSP60 ($F_{1,18} = 0.79$, $p = 0.39$) or HSP70 ($F_{1,18} = 1.10$, $p = 0.31$).

The mean level of HSP60 was negatively correlated with mean tarsus length on day 7 ($r_{18} = 0.61$, $p = 0.008$), but not with mean mass ($p > 0.10$). The same was true for mean H/L (tarsus length: $r_{17} = 0.83$, $p < 0.001$). The mean level of HSP60 was negatively correlated with mean nestling mass, tarsus length, and wing length on day 13 (Figure 2). The same was true for mean H/L (Figure 3). The mean level of HSP60 was positively correlated with SD in nestling mass and wing length on day 13 (Figure 4). This was not the case for mean H/L ($p > 0.10$). The regression models explained between 20% and 80% of variation in nestling morphological traits.

Discussion

Stress indicators to be used in field studies should be nondestructive, easy to obtain, and reliable. The H/L ratio has been shown to be a reliable stress indicator in field studies (Ots, Murumägi & Horak, 1998). Merino *et al.* (1998) have shown the levels of HSP60 to reflect the incidence of at least some stressors found in the wild. Here we show that both indicators are significantly related, strengthening the validity of their use for future field studies. The level of HSP70, which has been used in other studies of stress in wild birds (Merino *et al.*, 1999; Eeva *et al.*, 2000), did not correlate with the other two. This suggests that HSP70 is a poorer indicator of stress in birds, at least in the range of tissues and stressors tested. Both stress responses may be triggered by elevated levels of stress hormones, which are triggered by malnutrition in broods (Kitaysky *et al.*, 1999). Laboratory studies have detected that immunological challenges increase the concentration of specific stress proteins in some tissues (McComb & Spurlock, 1997), indicating that there is a link between the immune response and stress protein synthesis. The mechanistic basis for this link remains to be unravelled by physiologists and immunologists.
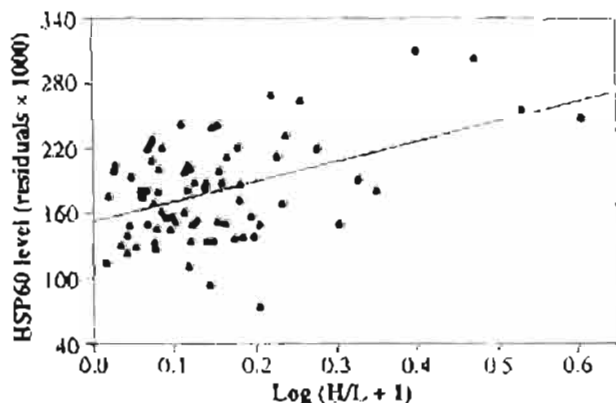

FIGURE 1. Correlation between levels of HSP60 and transformed H/L ratios for individual nestlings ($r_{61} = 0.52$, $p < 0.001$).
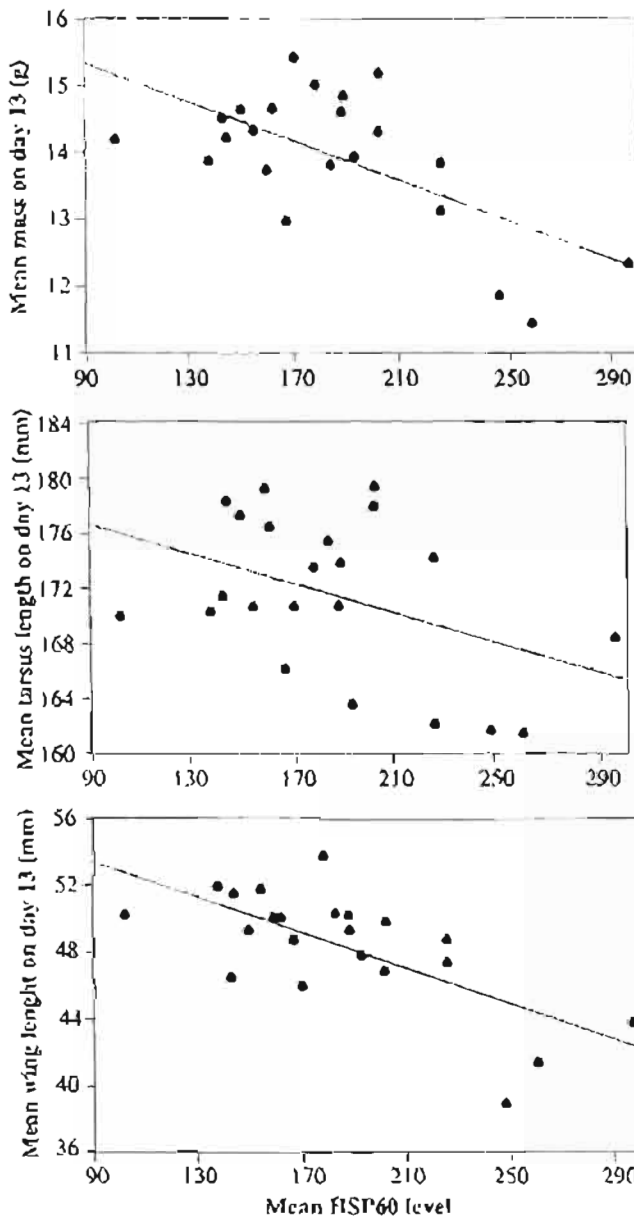
FIGURE 2. Correlations between mean HSP60 level and mean nestling mass (r_{22} = 0.64, p = 0.001), mean tarsus length (r_{22} = 0.43, p = 0.044), and mean wing length (r_{20} = 0.69, p < 0.001) on day 13.
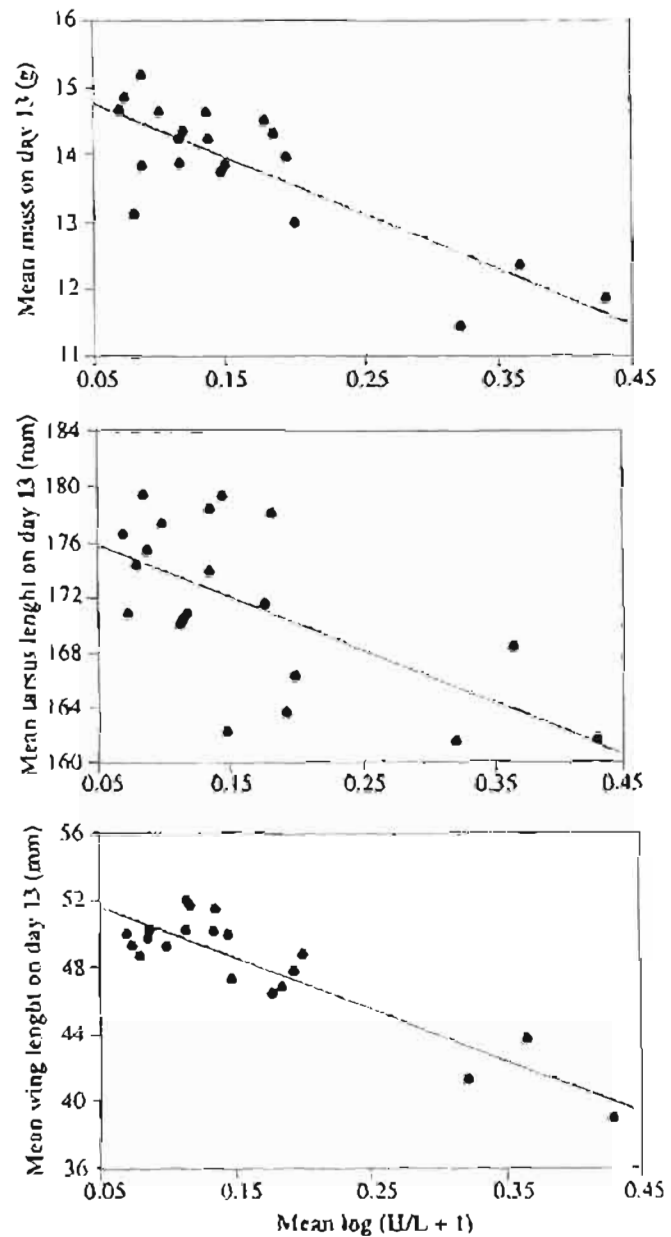

FIGURE 3. Correlations between mean transformed H/L and mean nestling mass (r_{20} = 0.82, p < 0.001), mean tarsus length (r_{20} = 0.62, p = 0.003), and mean wing length (r_{20} = 0.89, p < 0.001) on day 13.

Nestling growth showed a very close association with stress indicators. By day 7 of life, undersized nestlings already had clear symptoms of stress with respect to both indicators. Shortly before fledging, the fit of nestling mass and morphology to both H/L and HSP60 level was impressive. HSP70 again revealed itself as an unsuitable indicator of nutritional stress. H/L was clearly the index best explaining variation in mass and size, recommending it for future studies of nutritional stress. Tarsus length was the measure least responsive to stress, suggesting that it is more independent of environmental variation and more canalized by heritable factors (but see Moreno, Potti & Merino, 1997). This has been shown in other studies of passerines (Boag & van Noordwijk, 1987; Merilä & Gustafsson, 1993). The most stress-sensitive measure, wing length, has been shown to be

a good estimate of nutritional condition in nestling birds (Nowicki *et al.*, 2000). The fact that up to 80% of variation in nestling traits is explained by some stress indicator is clear evidence that there is huge environmental variation affecting growth patterns. Heritability of fitness-related traits like mass and wing length may become insignificant faced with such a massive impact of physiological stress, presumably related to nutrition (Alatalo, Gustafsson & Lundberg, 1990; Hoffmann & Merilä, 1999). This prediction is valid only under the assumption that stress affects all individuals similarly. Although proper cross-fostering experiments are needed to ascertain the heritability of morphological traits (Merilä, 1997), detecting stress-related variation may give a useful hint about the potential for selection in natural populations.
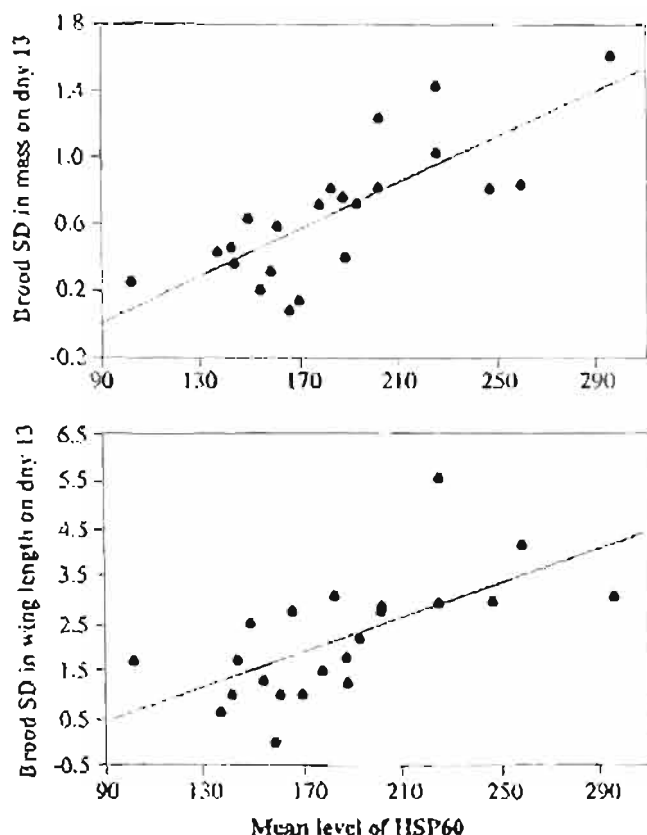
FIGURE 4. Correlations between mean HSP60 level and intra-brood SD in nestling mass ($r_{22} = 0.78$, $p < 0.001$) and in nestling wing length ($r_{22} = 0.65$, $p = 0.001$) on day 13.

The two stress indicators explained best different aspects of malnutrition in broods. While H/L showed strong associations with brood means, the level of HSP60 was a much better predictor of variance within broods. So, although both may reflect food restriction, H/L may be related to the effects of nutrition per se, while HSP60 would reflect the stress imposed by asymmetric sibling competition. Sibling competition may inflict other stresses besides the purely nutritional ones. Begging, although not especially energetically costly (Soler et al., 1999), may induce stress mediated by corticosterone (Nuñez de la Mora, Drummond & Wingfield, 1996; Kitaysky, Wingfield & Piatt, 2001). Activities related to adopting strategic positions in the nest may also be stressful, especially for the smallest siblings (Brzek & Konarzewski, 2001). These competitive activities would be reflected in increased size asymmetry in broods. Merino et al. (1998) also detected associations between increased HSP60 levels and greater variance within broods. The link between competitive stress and HSP60 synthesis remains to be explored further.

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