
Author(s)

J.V. BRIAN1*, J.J. AUGLEY2 AND V.A. BRAITHWAITE2

Title

Endocrine disrupting effects on the nesting behaviour of male three-spined stickleback Gasterosteus aculeatus L

Original Citation

Journal of Fish Biology, Volume 68, Number 6, June 2006 , pp. 1883-1890(8);

This version is available at:

http://dx.doi.org/10.1111/j.0022-1112.2006.01053.x

Title: Endocrine disrupting effects on the nesting behaviour of male three-spined stickleback *Gasterosteus aculeatus* L.

Running headline: Endocrine disrupting effects on nesting behaviour

J.V. BRIAN[1]*, J.J. AUGLEY[2] AND V.A. BRAITHWAITE[2]

[1] Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK.

[2] Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, EH9 3JT, UK.

*Corresponding author

Tel: 01895 266 264

Fax: 01895 274 348

Email: jayne.brian@brunel.ac.uk

Abstract

The analysis of patterns of temporal variability in the nesting behaviour of male threespined stickleback (*Gasterosteus aculeatus*) exposed to the synthetic oestrogen, 17*β*-ethinylestradiol, revealed immediate, but transient, treatment-related effects. Gluing frequency and time spent near nest were significantly reduced in exposed fish at the beginning of the experiment. The expression of these behaviours subsequently recovered and there was no effect of treatment on nest building success. The potential causes and implications of these findings are discussed.

Key words: endocrine disruption, nesting behaviour, spiggin, oestrogen, androgen.

A wide variety of chemicals that enter the aquatic environment are capable of disrupting normal endocrine function. Endocrine disrupting chemicals (EDCs) that interfere with sex hormone function are of particular concern, having been associated with adverse effects on the reproductive development and physiology of fish (e.g. Aravindakshan *et al*., 2004; Jobling *et al.,* 2002; Nash *et al.,* 2004; Vethaak *et al.,* 2005). Endocrine disrupting effects on reproductive behaviour have received less attention, although there is some evidence to suggest that EDCs can disrupt the expression of courtship behaviour. For example, Bayley *et al*. (1999) demonstrated that male guppies *Poecilia reticulata* (Peters) exhibited a reduction in the rate and intensity of sigmoid sexual displays following exposure to the natural oestrogen, 17*ß* oestradiol, and the oestrogen mimic, 4-*tert*-octylphenol. Similarly, Bjerselius *et al*. (2001) found that the frequency of following, pushing and courtship behaviours performed by male goldfish *Carassius auratus* L. was reduced by exposure to 17*ß* oestradiol. Estrogenic effects on behaviour have also been reported in Japanese medaka *Oryzias latipes* (Temminck and Schlegel) and mosquitofish *Gambusia holbrooki* (Girard) (Gray *et al.,* 1999; Doyle and Lim, 2002). Such effects are likely to be of ecological significance given that these behaviours play a crucial role in bringing together receptive mates and synchronising the release of gametes (Bjerselius *et al.,* 2001). Any impairment to the ability to perform these behaviours may therefore have implications for fitness and reproductive success.

The behavioural repertoire of the three-spined stickleback *Gasterosteus aculeatus* L. provides an interesting model for the analysis of endocrine-mediated effects as the male performs a range of reproductive behaviours in addition to courtship. The onset of these behaviours is triggered by an increase in temperature

and day length, which stimulates the production of androgen, primarily 11-ketoandrostenedione (11KA), in the male testes. 11KA is then converted extra-testicularly to 11-ketotestosterone (11KT). The resulting increase in plasma 11KT is associated with the onset of physiological changes, such as the development of nuptial colouration and kidney hypertrophy, in addition to changes in behaviour (Borg and Mayer, 1995). These are characterised by an increase in the expression of aggression as the male attempts to establish a territory. He then performs a range of nest building behaviours and, once this structure is complete, he engages in a range of ritualistic courtship behaviours to attract a mate. Following a successful spawning, the male adopts sole responsibility for the clutch and performs a range of paternal behaviours, which include fanning, cleaning and brood defence (Rowland, 1994). These processes are therefore critical to male stickleback in terms of fitness and reproductive success.

The androgen-dependence of these processes indicates that the reproductive system of the stickleback may be vulnerable to disruption by chemicals that interfere with endocrine function (Arcand-Hoy and Benson, 1998). This has been investigated by comparing the intensity of courtship and aggressive behaviour exhibited by male sticklebacks before and after exposure to the synthetic oestrogen, 17β-ethinylestradiol. A significant reduction in aggression was observed (Bell, 2001). Male sticklebacks injected with 17β estradiol have also been found to exhibit delayed nest building and reduced parental care (Wibe *et al.,* 2002). There have been further reports of effects on non-reproductive behaviours, such as foraging strategy and anti-predator response (Bell, 2004; Wibe *et al.,* 2001). Endocrine-mediated effects on the reproductive physiology of stickleback have also been reported, including the induction of spiggin (Jakobsson *et al*., 1999) in response to exogenous androgen stimulation (Katsiadaki *et*

al., 2002). Conversely, this process can be suppressed by exposure to oestrogen (Katsiadaki pers. com.). Spiggin synthesis is of functional significance, as this glue-like protein plays an important role in binding nesting materials and secure them to the substrate (Barber *et al.,* 2001). However, the ecological implications of endocrine-mediated effects on the synthesis of this protein are currently unknown.

The aim of this study was to look for evidence of endocrine-mediated effects on nest-related processes and to relate these to changes in nest building success, thereby helping to establish whether these effects are likely to pose a threat to wild fish populations. The nest is of major functional significance in the breeding system of male stickleback, both in terms of its role in courtship and parental care (Barber *et al.,* 2001). Any impairment to the process involved in the construction and defence of this structure is therefore likely to influence the ability of male sticklebacks to attract a mate and rear young, which has obvious implications in terms of their fitness and reproductive success.

Sticklebacks were sampled, with the permission of the water bailiff, from the Water of Leith at Whelpside, near Balerno, Midlothian, Scotland, from April-June 2003. This was a rural location where the risk of exposure to EDCs was considered to be minimal. Two sampling methods were employed: 1) hand-nets were dragged through the water as close to the banks as possible and 2) minnow-traps were set close to the banks and left for a maximum period of 24 hours. Approximately 60 fish were caught in total. These were placed in buckets containing aerated river water and transported by car to aquarium facilities at the University of Edinburgh. They were then transferred into glass stock tanks (30 x 40 x 40cm) at a density of approximately

12 fish per tank. Laboratory conditions consisted of a 16:8 light dark cycle and a temperature of 15°± 1°C. The fish were fed twice daily with both live and defrosted frozen bloodworms. As the fish came into breeding condition, males were identified through the development of nuptial coloration. Ten male fish were selected from the holding tanks at the beginning of each experiment and placed in individual observation tanks.

Ten identical observation glass tanks (30 x 20 x 22cm) were set up, each containing 10l of de-chlorinated tap water, 500g of gravel, 50g of sand in a glass Petri dish, a plastic plant and two hundred 6-cm long strands of black nylon thread for use as nest building material. Opaque polythene sheets were placed between the tanks to prevent the males from seeing each other. Each male was presented with a gravid female, which was suspended in a beaker of water, for 10-minutes each day. This provided a visual stimulus, which encouraged the male to engage in nesting activities (Barber *et al.,* 2001).

Half of the observation tanks were dosed with 17β-ethinylestradiol (EE2), which is the active component of the human contraceptive pill. EE2 is a potent and persistent synthetic oestrogen that is commonly found in the aquatic environment. It has been detected in sewage effluents at concentrations of up to 62ng/l and in surface waters at concentrations of up to 5ng/l (reviewed in Pawlowski *et al*., 2004). EE2 was dissolved in ethanol before being diluted in water and added to the tanks to give a nominal exposure concentration of 10 ng/l. This is at the upper end of the range of concentrations detected in the environment. Control tanks were dosed in the same manner with ethanol. Dosing was carried out semi-blind: the behavioural observer

knew only that odd and even numbered tanks had different treatments, but the identity of these was unknown.

Tanks were primed for three days before the start of the experiment. The water in each tank was then renewed immediately prior to the addition of the fish. A preliminary investigation into the change in the oestrogenicity of the tank water over time was carried out using a yeast oestrogen screen developed by Routledge and Sumpter (1996). This revealed that the half-life of EE2 in this system was approximately six days. Consequently, after six days, half of the water in each tank was removed and replaced with water containing one and a half times the desired concentration of EE2. This returned the overall concentration in the tank to 10ng/l. The exposure then continued for a further six days. This resulted in a pulsed system of exposure to EE2. Although this meant that the exposure conditions varied throughout the experiment, this system reflects the "real world" exposure situation, in which the exposure concentration varies with rainfall and the rate of chemical flow.

Three replicates of the experiment were run consecutively, involving the analysis of a total of 30 male fish (15 dosed and 15 control). At the end of each experimental replicate, the fish were sacrificed by over-anaesthesia in MS222, followed by decapitation. All the experimental work was carried out in accordance with Home Office procedures (licence number PL60/2954).

The behavioural observations were made during a single 10-minute period per tank on each day of the first exposure period (days one to six). The order in which the fish were observed was randomised daily using a 10-sided dice. All observations were

carried out within a period of between two to three hours, beginning at approximately 1100 hours each day. Four nest-related behaviours were recorded; gluing frequency, nest alteration frequency, time spent fanning nest and time spent within one body-length of the nest. Time spent fanning and time spent near nest were recorded cumulatively over each ten-minute observation period. At the end of each experimental replicate, the presence or absence of a nest was recorded. Fish that failed to build nests were excluded from the behavioural analyses.

Behavioural data were tested for normality and homogeneity of variance and were inverse square-root transformed when the assumptions for parametric analyses were not met. (Zeros were removed by adding 0.1 to the data prior to transformation.) The data were then analysed using repeated-measures ANOVA. The proportion of exposed and control fish that had built nests by the end of the exposure period was compared using a Chi-squared test.

Two mortalities occurred during the course of the first experimental replicate. It was not possible to determine the cause of death, although the loss of one fish from each treatment group suggests that the mortalities were not related to EE2 exposure. The loss of these two fish resulted in the comparison of observations made from 14 EE2-treated and 14 control fish during the course of three replicated experiments.

The frequency and duration of nesting building behaviours performed by fish in each treatment between days one to six is shown in Figure 1(A-D). There was no significant effect of treatment alone on the expression of these behaviours. In contrast, there was considerable variability in nesting behaviour over time and, out of

the four behaviours recorded, two revealed patterns that were associated with treatment-related effects. The frequency of gluing behaviours performed by all fish varied over time (ANOVA, $F_{5,105}$, $P<0.05$; Fig. 1A) and there was a significant interaction between observation number and treatment (ANOVA, $F_{5,105}$, $P<0.05$). This means that the pattern of temporal variability in gluing frequency varied with EE2 exposure. There was also a significant interaction between observation number and treatment for the time spent within one body length of the nest (ANOVA, $F_{5,105}$, $P<0.01$; Fig. 1B), which means that the pattern of temporal variability in the expression of this parameter also differed between treatments. The frequency of fanning varied significantly during the course of the observations (ANOVA, $F_{5,105}$, $P<0.01$; Fig. 1C) and exposed fish appeared to fan less frequently than the controls. However, there was no significant effect of treatment on the pattern of temporal variability (ANOVA, $F_{5,105}$, $P=0.61$). Nest alteration frequency did not vary throughout the exposure period (ANOVA, $F_{5,105}$, $P=0.59$) and there was no evidence of treatment-related effects (ANOVA, $F_{5,105}$, $P=0.25$; Fig. 1D).

At the end of the 12-day exposure period, 10 of the 14 exposed fish had built nests, compared to 13 of the 14 controls. The difference between the numbers of nests produced by the fish in each treatment group was not statistically significant ($\chi^2_1=2.91$, $P=0.13$).

The results of this study revealed that EE2 treatment was associated with significant, but short lived, effects on the expression of nesting behaviour. This was evident from patterns of temporal variability in the gluing frequency and the time that males spent near their nests. Previous research on the endocrinology of the stickleback

has highlighted the significance of androgenic hormones in the control of male reproductive behaviours. For example, the removal of the male testes, which is the primary site of 11KA synthesis, has been found to abolish courtship, aggression and nesting behaviours (Borg and Mayer, 1995). Conversely, the expression of these behaviours can be fully reinstated by implanting castrated fish with capsules containing 11KA or 11KT (Borg and Mayer, 1995). This indicates that any effects of EE2 may be mediated via an indirect effect on the levels of endogenous androgen. This is consistent with the theory proposed by Bell (2001), who attributed the reduced aggression of male stickleback exposed to 15ng/l EE2 to the down-regulation of androgen synthesis by the actions of exogenous oestrogen. Although the study by Bell did not provide any evidence of oestrogenic effects on the levels of 11KT, this theory remains the most plausible explanation for the treatment-related effects reported here.

The effect of treatment on the expression of gluing behaviours and the amount of time spent near the nest varied throughout the period of exposure. Examination of the patterns of temporal variability revealed that the treatment-related effects appeared within 24 hours of exposure to EE2. This indicates that nesting behaviours respond rapidly to endocrine-mediated effects. However, these responses were short lived. The subsequent recovery in the expression of nest-related behaviours with time may reflect the degradation of EE2 in the tank water or the up-regulation of detoxification processes in exposed fish. The effects of this were evident by day 3 of the exposure, by which time, the intensity of nest-related behaviours performed by exposed fish often exceeded that of the controls. This indicates that the expression of nesting behaviours underwent a full recovery during the 6-day exposure period.

As yet, few studies have considered the effects of EDCs on behaviour over time, focusing instead on the analysis of effects at one or two time points or averaged over a series of time points (e.g. Bell, 2001; 2004; Wibe *et al.,* 2001; 2002). However, this approach fails to take account of the influence of temporal variations, thereby reducing the probability of detecting effects that are associated with treatment. The repeated measures analysis offers a more sensitive method of analysing this type of data, as it has the capacity to separate the effects of time and treatment, as well as to explore interactions between them. However, it is important to recognise that the short-term nature of this investigation prevented the analysis of chronic behavioural effects that may have become apparent over a more extended period of time.

The immediate, but transient, nature of the treatment-related effects that were detected in this study was manifested as a lag in the expression of nesting behaviours at the start of the exposure period. This finding is consistent with previous evidence that male stickleback injected with 17β oestradiol start to build nests significantly later than control fish (Wibe *et al.,* 2002). However, these behavioural perturbations were not associated with treatment-related effects on the proportion of fish that had built nests by the end of the experiment. Again, this was consistent with the findings of Wibe *et al.* (2002). This indicates that endocrine-mediated effects on nest-related processes do not have a significant influence on nest building success.

In summary, EE2 treatment was associated with significant effects on the nesting behaviour of male stickleback. This is consistent with previous reports that oestrogen affects the expression of aggressive behaviour and parental care (Bell, 2001; Wibe *et al.,* 2002). However, the effects were both subtle and short-lived and

did not appear to influence nest building success. This indicates that endocrine-mediated effects on nest-related processes are unlikely to be of ecological significance when exposures are limited in duration and do not exceed the effective concentration tested here. However, wild fish that are exposed to the combined effects of multi-component mixtures of EDCs for extended periods of time are likely to be more susceptible to oestrogenic effects (Brian *et al.,* 2005). This suggests that it may be pertinent to investigate effects on nest building over an extended period of exposure. It should also be noted that the influence of EDCs on nest building success may be quite different in real exposure situations, where males have to compete for territories and nesting materials. Further consideration of these issues will be required to determine the possible fitness implications of the endocrine-mediated behavioural effects observed in this study.

We thank Carolyn Gardner for help with fish maintenance, John Sumpter for providing the recombinant yeast cells, Cath Harris for advice on use of the YES and Nick Colgrave for advice on the statistical analyses. This work was funded by a British Ecological Society Project Grant (No. 1982) awarded to JVB. VAB thanks The Leverhulme Trust for continued financial support.

References

Aravindakshan, J., Paquet, V., Gregory, M., Dufresne, J., Fournier, M., Marcogliese, D.J. & Cyr, D.G. (2004). Consequences of xenoestrogen exposure on male reproductive function in Spotted Shiners (*Notropis hudsonius*). *Toxicological Sciences* **78**, 156-165

Arcand-Hoy, L.D. & Benson, W. (1998). Fish reproduction: an ecologically relevant indicator of endocrine disruption. *Environmental Toxicology and Chemistry* **17**, 49-57.

Barber, I., Nairn, D. & Huntingford, F.A. (2001). Nests as ornaments: revealing construction by male sticklebacks. *Behavioural Ecology* **12**, 390-396.

Bayley, M., Nielsen, J.R. & Baatrup, E. (1999). Guppy sexual behavior as an effect biomarker of oestrogen mimics. *Ecotoxicology and Environmental Safety* **43**, 68-73.

Bell. A.M. (2001). Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus. Animal Behaviour* **62**, 775-780.

Bell, A.M. (2004). An endocrine disruptor increases growth and risky behavior in threespined stickleback (*Gasterosteus aculeatus*). *Hormones and Behaviour* **45**, 108-114.

Bjerselius, R., Lundstedt-Enkel, K., Olsen, H., Mayer, I. & Dimberg, K. (2001) Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17 beta-estradiol. *Aquatic Toxicology* **53**, 139-152.

Borg, B. & Mayer, I. (1995). Androgens and behaviour in the three-spined stickleback. *Behaviour* **132**, 1025-1035.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, P., Jonkers, N., Bonfa, A., Marcomini, A. & Sumpter, J.P. (2005). Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environmental Health Perspectives* **113**, 721-728.

Doyle, C.J. & Lim, R.P. (2002) The effect of 17 beta-estradiol on the gonopodial development and sexual activity of *Gambusia holbrooki*. *Environmental Toxicology and Chemistry* **21**, 2719-2724.

Gray, M.A., Teather, K.L. & Metcalfe, C.D. (1999). Reproductive success and behavior of Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environmental Toxicology and Chemistry* **18**, 2587-2594.

Jakobsson, S., Borg, B., Haux, C. & Hyllner, S.J. (1999). An 11-ketotestosterone induced kidney-secreted protein: the nest building glue from male three-spined stickeback, *Gasterosteus aculeatus*. *Fish Physiology and Biochemistry* **20**, 79-85

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R. & Sumpter, J.P. (2002). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology Reproduction* **67**, 515-524.

Katsiadaki, I., Scott, A.P. & Mayer, I. (2002). The potential of the three spined stickleback as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental Research* **54**, 725-728.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmit-Allner, P. & Tyler, C.R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes

reproductive failure in fish. *Environmental Health Perspectives* **112**,1725-1733.

Pawlowski, S., van Aerle, R., Tyler, C.R. & Braunbeck, T. (2004). Effects of 17 alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety* **57**, 330-345.

Routledge, E.J. & Sumpter, J.P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Science and Technology* **15**, 241-248.

Rowland, W.J. (1994). Proximate determinants of stickleback behaviour: an evolutionary perspective. In *The Evolutionary Biology of the Threespine Stickleback* (Bell, M.A., and Foster, S.A., eds), pp297-344. Oxford University Press, New York.

Vethaak, A.D., Lahr, J. Schrap, S.M., Belfroid, A.C., Rijs, G.B.J., Gerritsen, A., de Boer, J., Bulder, A.S., Grinwis, G.C.M., Kuiper, R.V., Legler, J., Murk, T.A.J., Peijnenburg, W., Verhaar, H.J.M. & de Voogt, P. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere* **59**, 511-524

Wibe, A.E., Rosenqvist, G. & Jenssen, B.M. (2002). Disruption of male reproductive behavior in threespine stickleback *Gasterosteus aculeatus* exposed to 17 beta-estradiol. *Environmental Research* **90**, 136-141.

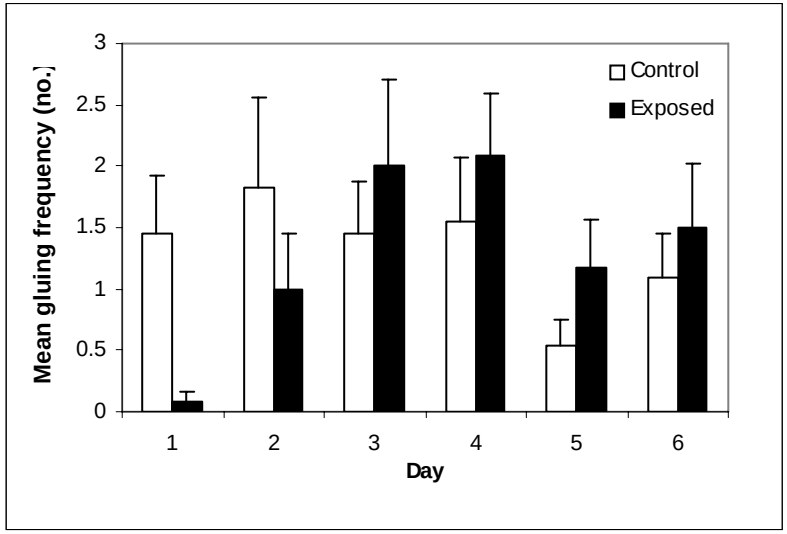
Wibe, A.E., Nordtug, T. & Jenssen, B.M. (2001). Effects of bis(tributyltin)oxide on antipredator behavior in threespine stickleback *Gasterosteus aculeatus* L. *Chemosphere* **44**, 475-481.

Figure 1A. Variations in gluing frequency during the exposure period. Error bars represent one S.E. of the mean.

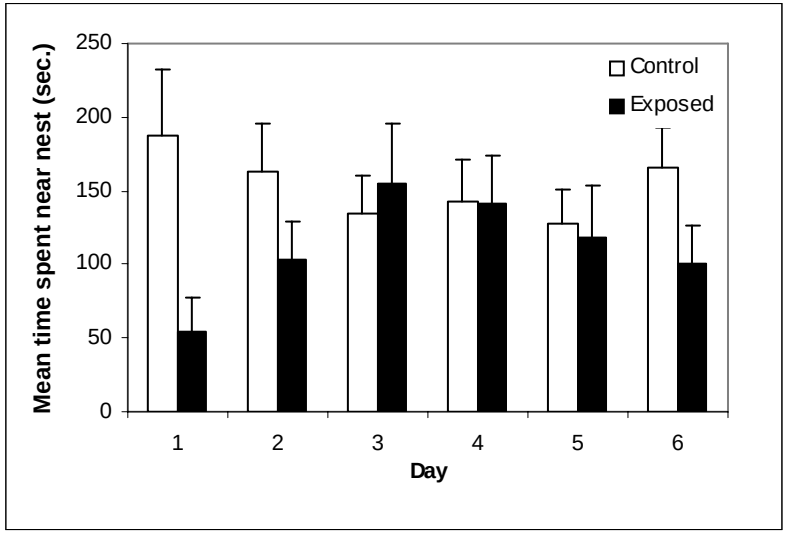
Figure 1B. Variations in the time spent within one body length of the nest during the exposure period. Error bars represent one S.E. of the mean.

Figure 1C. Variations in the time spent fanning during the exposure period. Error bars represent one S.E. of the mean.

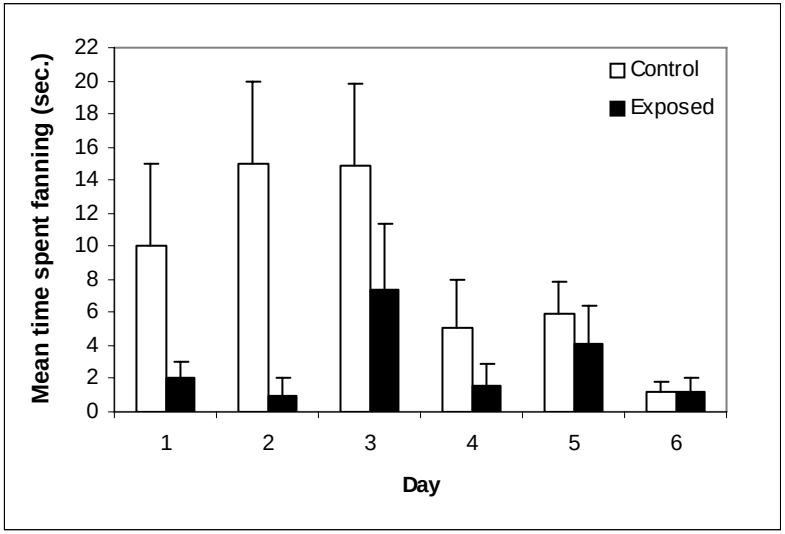
Figure 1D. Variations in the frequency of nest alterations during the exposure period. Error bars represent one S.E. of the mean.


Mean gluing frequency (no.)
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
Day
Control
Exposed

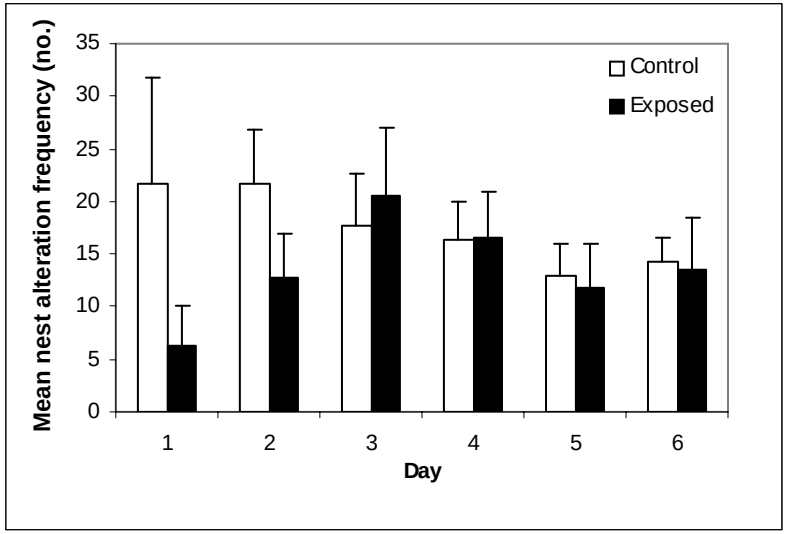
1A


Mean time spent near nest (sec.)
250
200
150
100
50
0
1
2
3
4
5
6
Day
Control
Exposed

1B


Mean time spent fanning (sec.)
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
Day
Control
Exposed

1C


Mean nest alteration frequency (no.)
35
30
25
20
15
10
5
0
1
2
3
4
5
6
Day
Control
Exposed

1D